Sensitisation of the trigeminovascular system;

Implications for migraine pathophysiology.

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ABSTRACT

Migraine is an episodic brain disorder characterised often by attacks of throbbing head pain and in many patients by sensitisation of the facial skin. Current research indicates that the dural blood vessels and their neural connectivity with the trigeminocervical complex play a fundamental role in the perception of these symptoms. As such, characterisation of this pathway may provide an insight into the pathological processes occurring during a migraine attack and offer new targets for the development of novel anti-migraine treatment strategies.

Utilising the techniques of *in vivo* electrophysiology, intravital microscopy and *c-fos* immunohistochemistry, the studies in this thesis have explored the consequences of trigeminal primary afferent neuronal activation during the following conditions:

-Exposure to glyceryl trinitrate, a nitric oxide releasing compound, known to induce migraine in susceptible individuals.

-Peripheral sensitisation following application of various mediators to the dural and facial receptive field.

-Following "wind-up" stimuli, known to hyper-excite spinal neurons

The findings from these studies demonstrate that the behaviour of second order neurons in the spinal trigeminal nucleus is fundamentally different following input from the dura mater compared to input from facial cutaneous afferents. Furthermore, the time course of sensitisation seen following application of prostaglandin E_2 to the dural receptive field correlates well to the allodynia and hyperalgesia reported during migraine. Sensitisation of dural afferents may thus underlie at least part of the pathophysiology associated with migraine.

TABLE OF CONTENTS

TABLE OF CONTENTS ..... - 3 -

ABBREVIATIONS ..... - 11 -

ACKNOWLEDGEMENTS ..... - 12 -

GENERAL INTRODUCTION ..... - 13 -

1.1 The history of headache ..... *- 13 -*

1.2 Migraine ..... *- 14 -*

1.2.1 Migraine Prevalence ..... - 18 -

1.2.2 Social impact of migraine ..... - 20 -

1.3 Anatomy of the trigeminovascular system ..... *- 20 -*

1.3.1 The Trigeminal nerve ..... - 21 -

1.3.2 Morphology of nerve fibres innervating the dura mater ..... - 23 -

1.3.2.1 Sensory innervation ..... - 23 -

1.3.2.2 Autonomic innervation of dural and intracranial vessels ..... - 23 -

1.3.3 Primary afferent cell bodies in the trigeminal system ..... - 24 -

1.3.4 The Dura mater ..... - 25 -

1.3.5 The cerebral vasculature ..... - 25 -

1.3.5.1 Intracranial vessels ..... - 26 -

1.3.5.2 Extracranial arteries ..... - 27 -

1.3.6 Trigeminal brainstem nuclear complex ..... - 27 -

1.3.6.1 Characterisation of second order neurons in the trigeminal brainstem nuclear complex ...- 32 -

1.4 Transmitters in the Trigeminovascular system ..... *- 33 -*

1.4.1 Calcitonin gene related peptide (CGRP) ..... - 33 -

1.4.2 Substance P ..... - 34 -

1.4.3 Serotonin ..... - 35 -

1.4.4 Other transmitters modulating VBNC activity ..... - 36 -

1.5 Modulation of neuronal processing in the Trigeminal brainstem nuclear complex ... *- 37 -*

1.5.1 Neuronal modulation within the Trigeminal brainstem nuclear complex ..... - 37 -

1.5.2 Higher centre connections with the trigeminal nuclear brainstem complex ..... - 39 -

1.5.2.1 Ascending Spinal and Trigeminal tracts ..... - 39 -

1.5.2.2 Descending Spinal and Trigeminal tracts ..... - 41 -

1.5.2.3 Thalamus ..... - 43 -

1.5.2.4 Hypothalamus ..... - 43 -

1.5.2.5 Periaqueductal grey ..... - 44 -

1.5.2.6 Nucleus raphe magnus ..... - 46 -

1.5.2.7 Gigantocellular neurons of the reticular area ..... - 47 -

1.5.2.8 Locus ceruleus ..... - 48 -

1.5.2.9 Parabrachial area ..... - 49 -

1.5.3 Diffuse noxious inhibitory controls ..... - 51 -

1.5.4 Autonomic connections with the trigeminovascular system ..... - 51 -

1.6 Visceral pain ..... *- 52 -*

1.7 Migraine Models ..... *- 53 -*

1.7.1 Neuronal sensitisation as a model of migraine pain ... - 54 -
1.7.2 Electrophysiology ... - 58 -
1.7.2.1 Human studies ... - 58 -
1.7.2.2 Animal studies ... - 59 -
1.7.3 Neuropeptide release ... - 59 -
1.7.4 Plasma protein extravasation (PPE) and neurogenic inflammation ... - 60 -
1.7.5 Intravital microscopy ... - 61 -
1.7.6 Molecular markers ... - 62 -
1.7.7 Brainstem manipulations ... - 65 -
1.7.8 Models of aura- cortical spreading depression (CSD) ... - 65 -
1.7.9 Functional imaging ... - 66 -
1.8 Drug treatments for migraine ... - 67 -
1.8.1 Pre-emptive treatment ... - 68 -
1.8.2 Anti-Emetics ... - 68 -
1.8.3 Acute treatment ... - 69 -
1.8.3.1 Ergot Alkaloids ... - 69 -
1.8.3.2 Triptans ... - 70 -
1.8.3.3 Non-steroidal anti-inflammatory drugs ... - 72 -
1.8.3.4 Opioids ... - 73 -
1.8.3.5 Miscellaneous drugs ... - 74 -
1.8.4 Prophylactics ... - 75 -
1.8.4.1 β-blockers ... - 75 -
1.8.4.2 Anti-convulsants ... - 76 -
1.8.4.3 Serotonin antagonists ... - 76 -
1.8.4.4 Antidepressants ... - 76 -
1.8.4.5 Botulinum toxin A ... - 77 -
1.8.4.6 Miscellaneous drugs ... - 78 -
1.9 Genetics of Migraine ... - 78 -
1.10 Summary & Aims ... - 81 -
1.10.1 Study 1 –GTN ... - 81 -
1.10.2 Study 2-Prostaglandins ... - 82 -
1.10.3 Study 3-Wind-up ... - 83 -
2 METHODS ... - 85 -
2.1 Electrophysiology and Intravital microscopy ... - 85 -
2.1.1 Preparatory surgery and anaesthesia ... - 85 -
2.1.1.1 Exposure of the middle meningeal artery ... - 87 -
2.1.1.2 Exposure of the trigeminal nucleus caudalis ... - 87 -
2.1.2 Intravital microscopy ... - 87 -
2.1.2.1 Data measurement ... - 88 -
2.1.2.2 Analysis of data ... - 88 -
2.1.3 Electrophysiology ... - 89 -
2.1.3.1 Data capture ... - 92 -
2.1.3.2 Experimental protocols ... - 96 -
2.1.3.3 Analysis of data ... - 97 -

2.2 Immunohistochemistry ..... *- 97 -*
2.2.1 Animal preparation ..... - 97 -
2.2.2 Tissue processing ..... - 98 -
2.2.2.1 Cell counting ..... - 101 -
3 STUDY 1- MODULATION OF THE TRIGEMINOVASCULAR SYSTEM WITH GLYCERYL TRINITRATE BEFORE AND AFTER THE INDUCTION OF CENTRAL SENSITISATION ..... - 102 -
3.1 Introduction ..... *- 102 -*
3.1.1 Biological effects of GTN ..... - 102 -
3.1.2 Central sensitisation and GTN ..... - 104 -
3.2 Methods ..... *- 107 -*
3.2.1 Intra-arterial infusions and topical administration of GTN ..... - 107 -
3.2.1.1 Intravital microscopy ..... - 107 -
3.2.1.2 Electrophysiology ..... - 110 -
3.2.2 Intravenous infusions of GTN following induction of central sensitisation ..... - 110 -
3.2.2.1 Electrophysiology ..... - 110 -
3.2.3 Immunohistochemistry ..... - 111 -
3.2.3.1 Experimental protocol using formalin as a mediator of central sensitisation ..... - 111 -
3.2.3.2 Experimental protocol using capsaicin as a mediator of central sensitisation ..... - 112 -
3.2.3.3 Experimental protocol using Prostaglandin E_2 (PGE_2) and Nerve Growth Factor (NGF) as a mediator of central sensitisation ..... - 113 -
3.2.3.4 Statistical analysis ..... - 113 -
3.3 Results ..... *- 115 -*
3.3.1 Intravital Microscopy ..... - 116 -
3.3.1.1 Intra-arterial infusion ..... - 116 -
3.3.1.2 Topical GTN administration ..... - 119 -
3.3.2 Electrophysiology ..... - 121 -
3.3.2.1 Intra-arterial GTN infusions ..... - 121 -
3.3.2.2 Topical GTN application over the MMA ..... - 124 -
3.3.3 Intravenous GTN infusions ..... - 124 -
3.3.3.1 Formalin induced sensitisation ..... - 124 -
3.3.3.2 Capsaicin induced sensitisation ..... - 128 -
3.3.3.2.1 Topical capsaicin application over the MMA ..... - 128 -
3.3.3.2.2 Topical capsaicin application over the MMA and GTN ..... - 128 -
3.3.3.2.3 Injection of capsaicin in the FRF ..... - 129 -
3.3.3.2.4 The effect of capsaicin on FRF stimulation ..... - 131 -
3.3.4 Immunohistochemistry ..... - 133 -
3.3.4.1 Formalin ..... - 134 -
3.3.4.2 Capsaicin ..... - 137 -
3.3.4.3 PGE_2 and NGF ..... - 141 -
3.4 Discussion ..... *- 142 -*
3.4.1 Intra-arterial GTN infusions ..... - 142 -
3.4.2 The effect of GTN in combination with inflammatory mediators ..... - 145 -
4 STUDY 2 THE ROLE OF PROSTANOIDS IN SENSITISATION OF THE TRIGEMINOVASCULAR SYSTEM .- 151 -

4.1 Introduction ..... *- 151 -*
4.2 Methods ..... *- 163 -*
4.2.1 Receptive field characterisation ..... - 163 -
4.2.2 Evaluation of the sensitising effects of PGE_2 ..... - 164 -
4.2.3 Naratriptan inhibition of PGE_2 sensitised responses ..... - 165 -
4.2.4 Prostanoid receptor subtypes involved in PGE_2- sensitisation ..... - 165 -
4.2.4.1 EP_2 agonist ..... - 165 -
4.2.4.2 EP_1 antagonist ..... - 165 -
4.2.4.3 EP_4 antagonist ..... - 166 -
4.2.5 Statistical analysis ..... - 166 -
4.3 Results ..... *- 167 -*
4.3.1 Topical PGE_2 application to MMA ..... - 168 -
4.3.2 Effect of Topical PGE_2 on responses evoked by stimulation of the FRF ..... - 175 -
4.3.3 Naratriptan experiments ..... - 177 -
4.3.4 Administration of PGE_2 into the FRF ..... - 179 -
4.3.5 Intra-arterial administration of butaprost and PGE2 ..... - 179 -
4.3.6 Topical application of butaprost ..... - 180 -
4.3.7 GW627368 and ZM325802 ..... - 182 -
4.4 Discussion ..... *- 188 -*
5 STUDY 3-EXPLORATION OF THE CHARACTERISTICS OF VC NEURONS WITH RELEVANCE TO PRIMARY AFFERENT INPUT ..... - 197 -
5.1 Introduction ..... *- 197 -*
5.2 Methods ..... *- 201 -*
5.2.1 Wind-up of Vc cells using dural stimulation ..... - 201 -
5.2.1.1 Characterisation of Aδ and C-fibre responses ..... - 203 -
5.2.2 Wind-up of Vc cells using facial stimulation ..... - 203 -
5.2.3 The effect of MK-801, an NMDA antagonist, on MMA-evoked Vc responses ..... - 204 -
5.2.4 Wind-up of Vc cells following the induction of central sensitisation ..... - 204 -
5.2.4.1 Data analysis ..... - 205 -
5.3 Results ..... *- 206 -*
5.3.1 Wind-up of cells receiving a dural input ..... - 206 -
5.3.1.1 Aδ response ..... - 206 -
5.3.1.2 C-fibre response ..... - 207 -
5.3.1.3 Brush-pinch responses ..... - 208 -
5.3.2 Wind-up of cells following electrical stimulation of the FRF ..... - 208 -
5.3.2.1 A-δ responses ..... - 209 -
5.3.2.2 C-fibre responses ..... - 210 -
5.3.2.3 Brush-pinch responses ..... - 214 -
5.3.3 MK-801 ..... - 214 -
5.3.4 Mustard Oil (MO) stimulation ..... - 216 -
5.4 Discussion ..... *- 223 -*
6 GENERAL DISCUSSION ..... - 232 -
7 APPENDIX ..... - 245 -
7.1 Appendix A-Drugs and dilutions ..... *- 245 -*

7.1.1 Anaesthetics ..... - 245 -
7.1.2 Study 1 test compounds ..... - 246 -
7.1.3 Immunohistochemical reagents ..... - 247 -
7.1.4 Study 2 ..... - 248 -
7.1.5 Study 3 ..... - 249 -
7.2 Appendix B Analysis of Electrophysiological data ..... *- 251 -*
7.2.1 Sequence files ..... - 251 -
7.2.1.1 50 x 1Hz ..... - 251 -
7.2.2 On line analysis of neuronal responses ..... - 253 -
7.2.2.1 Script file ..... - 253 -
7.3 Appendix C Manufacturers and suppliers ..... *- 257 -*
8 BIBLIOGRAPHY ..... - 259 -

List of Figures

Figure 1. The Trigeminovascular system ..... - 21 -
Figure 2. The innervation territories of the trigeminal nerve ..... - 22 -
Figure 3. The Trigeminal Brainstem Nuclear Complex (VBNC) ..... - 29 -
Figure 4. Schematic representation of "gate control" ..... - 38 -
Figure 5. Ascending spinal trigeminal tracts conveying pain information ..... - 40 -
Figure 6 Analgesia system of the brain & spinal cord ..... - 42 -
Figure 7. Brainstem structures with trigeminal brainstem nuclear complex connections ..... - 50 -
Figure 8 c-Fos expression ..... - 64 -
Figure 9 Electrophysiological recording system ..... - 92 -
Figure 10 Window discriminator to isolate single units ..... - 94 -
Figure 11 Labelling of c-fos ..... - 100 -
Figure 12 c-fos immunoreactivity in the Vc ..... - 101 -
Figure 13 Possible sites of action of GTN ..... - 103 -
Figure 14 Intravital microscopy-experimental protocol ..... - 108 -
Figure 15 Topical application of GTN over the closed cranial window ..... - 109 -
Figure 16 Dilatation of the MMA following GTN ..... - 120 -
Figure 17 Effect of intra-arterial GTN infusion on evoked activity ..... - 123 -
Figure 18 Formalin (5%) into the FRF generates pressor effects on blood pressure ..... - 127 -
Figure 19 Mean normalised evoked responses following capsaicin ..... - 130 -
Figure 20 Response to facial receptive field stimulation following capsaicin ..... - 132 -
Figure 21 c-fos immunohistochemistry observed in the Vc ..... - 133 -
Figure 22 Total c-fos expression following GTN and capsaicin combinations ..... - 138 -
Figure 23 Facilitation by GTN of c-fos expression induced by capsaicin ..... - 140 -
Figure 24 c-fos expression following administration of PGE_2, GTN and NGF ..... - 141 -
Figure 25 Site of action of GTN ..... - 144 -
Figure 26 Topical capsaicin application results in central sensitisation ..... - 146 -
Figure 27 Liberation of AA from cell membranes by the action of phospholipases ..... - 152 -
Figure 28 Prostaglandin synthesis ..... - 155 -
Figure 29 Peripheral sensitisation following PG release ..... - 160 -
Figure 30 Neuronal responses to electrical stimulation of the MMA, before and after PGE_2 application ..... - 169 -
Figure 31 Post-stimulus histograms of electrically evoked responses ..... - 171 -
Figure 32 Effect of PGE_2 applied to the MMA on neuronal activity ..... - 172 -
Figure 33 Effect of PGE_2 applied to the MMA on spontaneous neuronal activity ..... - 174 -
Figure 34 Response to FRF stimulation after PGE_2 application ..... - 176 -
Figure 35 Effect of naratriptan of MMA-evoked responses in the presence of PGE_2 ..... - 178 -
Figure 36 Comparison of the effect of butaprost and PGE_2 following intra-arterial infusion and topical application over the MMA ..... - 181 -
Figure 37 Effect of the EP_1 and EP_4 antagonist on PGE_2 sensitisation ..... - 185 -
Figure 38 EP_1 and EP_4 antagonists do not effect un-sensitised MMA evoked firing ..... - 187 -
Figure 39 Brush responses of the FRF ..... - 202 -
Figure 40 C-fibre response following stimulation of the face ..... - 211 -

Figure 41 Wind-up increases during the experiment ..... - 213 -
Figure 42 Effect of MK-801 on MMA-evoked Vc responses ..... - 215 -
Figure 43 Effect of topical MO application on MMA-evoked Vc responses ..... - 217 -
Figure 44 Expansion of FRF's following MO ..... - 220 -
Figure 45 Response to FRF stimulation following MO application ..... - 222 -

List of Tables

Table 1 Primary headache syndromes defined by IHS (1988) ..... - 15 -
Table 2 Summary of some of the studies examining migraine prevalence in different geographical populations ..... - 19 -
Table 3. Main cranial structures resulting in pain perception as identified by Ray and Wolff (1940) .- 26 -
Table 4 Migraine triggers ..... - 68 -
Table 5 Summary of the effects of GTN infusion on MMA diameter ..... - 118 -
Table 6 Effects of GTN on MMA-evoked, Vc responses ..... - 122 -
Table 7 Summary of the maximum responses seen following formalin & GTN ..... - 126 -
Table 8 Total mean fos counts following treatment with formalin and GTN ..... - 135 -
Table 10 Results summary of study 1 ..... - 150 -
Table 11 Ligand availability for the EP receptors ..... - 157 -
Table 12 Effects of EP_1 and EP_4 antagonists on PGE_2 sensitisation of Vc responses ..... - 184 -
Table 13 Duration of MO effect ..... - 218 -
Table 14 Central action of prostaglandins ..... - 243 -
Table 15 Manufacturers and Suppliers of equipment used in these studies ..... - 258 -

Abbreviations

ASA	Acetylsalicylic acid
C_1	Spinal cord, cervical division 1
C_2	Spinal cord, cervical division 2
CGRP	Calcitonin gene-related peptide
FRF	Facial receptive field
GABA	gamma-aminobutyric acid
GTN	Glyceryl trinitrate
IHS	International Headache Society
5-HT	5-hydroxytryptamine
MMA	Middle meningeal artery
MO	Mustard oil
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
NS	Nociceptive specific
PAG	Periaqueductal grey
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGI_2	Prostaglandin I_2
PPE	Plasma protein extravasation
SP	Substance P
SSS	Superior sagittal sinus
VBNC	Trigeminal brainstem nuclear complex
V_1	First (ophthalmic) division of the trigeminal nerve
V_2	Second (maxillary) division of the trigeminal nerve
V_3	Third (mandibular) division of the trigeminal nerve
Vc	Trigeminal nucleus caudalis
Vi	Trigeminal nucleus interpolaris
Vo	Trigeminal nucleus oralis
Vsp	Spinal trigeminal nucleus
WDR	Wide dynamic range

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General Introduction

1.1 The history of headache

Recorded accounts of the disabling nature of head pain are first documented in transcribed ancient Egyptian medical papers, which are estimated to date back as far as 3000BC. Hippocrates, 2460 years ago, clearly emphasised the visual disturbance, nausea, and vomiting associated with unilateral head-pain (Critchely, 1967) that was different in character to other forms of headache (Adams, 1939). Despite observations of migraine-like symptoms in these early times that are in extremely good agreement with the official current classification of the disease, few hypotheses of the origin of migraine pain are recorded until the 17th century.

Dr Willis' *Practice of Physicke* published in 1684 and reproduced in 1963 (Knapp) states that *"the source of pain is not the brain, cerebellum or medulla" but rather "distension of the vessels which pull the nervous fibres one from another and so brings to them painful corrugations or wrinklings"*. The theory that cranial vessels were the source of abnormality underlying migraine was further added to by the observation of Dubois-Reymond in 1859 that the pain of migraine "*mounts synchronously with the pulse of the temporal artery*" but ceases on compression of the carotid artery (Schiller *et al.*, 1975). The nineteenth century also saw suggestions of a central neuronal component of migraine with Living's analogy with epilepsy (1873) and Hughling Jackson's (1880) description of migraine as "a form of sensory epilepsy with headache and vomiting as an epiphenomenon" (Schiller 1975). Over 100 years later, there is still considerable debate between researchers whether migraine results from a central or

peripheral abnormality. This thesis thus explores the basis of the neuronal dysfunction in the trigeminal system that may account for the dural symptoms observed over many centuries.

1.2 Migraine

Headache is among the most common complaints in the western world. In many cases, headache symptoms represent primary headache syndromes such as migraine, tension-type headache and cluster headache. However, headache symptoms may also be an indication of a "secondary headache" resulting from underlying illness such as infection, cerebral haemorrhage, brain tumour and meningitis. Hence, accurate diagnosis of headache type is essential both in excluding serious illness and in determining treatment. Before the 1960's, the definition of headache was not standardised and no internationally accepted classification system existed. In 1962, an Ad Hoc Committee of the National Institute of Health, published the first international classification system (The Ad Hoc Committee on the Classification of Headache; Friedman *et al.*, 1962), however many felt that the classification required too much subjective interpretation. As a result, in 1985, a committee of world headache experts undertook the mammoth task of drawing a classification system based on clear operational criteria. The publication in 1988 of this classification system by the International Headache Society (IHS) not only provided a template for clinical diagnosis, but was a huge aid for researchers in the focusing of specific symptoms associated with headache types, allowing well defined patient populations for study (Headache classification Committee of the International Headache Society, 1988). The classification of the primary headaches, as defined by the IHS is summarised below,

although some changes to this are expected soon following the first revision of the criteria.

Primary headache disorders
1. Migraine
1.1 Migraine without aura (Common migraine)
1.2 Migraine with aura (Classical migraine)
1.3 Ophthalmoplegic
1.4 Retinal migraine
1.5 Childhood periodic syndromes that may be precursors to or associated with migraine
1.6 Complications of migraine
1.7 Migrainous disorder not fulfilling above criteria
2. Tension-type headache
2.1 Episodic
2.2 Chronic
2.3 Headache not fulfilling above criteria
3. Cluster headache and chronic paroxysmal hemicrania
3.1 Cluster headache
3.1.1 Periodicity undetermined
3.1.2 Episodic
3.1.3 Chronic
3.2 Chronic paroxysmal hemicrania
3.3 Cluster headache-like disorder not fulfilling above criteria
4. Miscellaneous headaches un-associated with structural lesions
4.1 Idiopathic stabbing headache
4.2 External compression headache
4.3 Cold stimulus headache
4.4 Benign cough headache
4.5 Benign exertional headache
4.6 Headache associated with sexual activity

Table 1 Primary headache syndromes defined by IHS (1988)

This thesis is concerned with understanding dural and cranial neurovascular nociception that may be relevant to migraine. For the purpose of research, it is useful to consider migraine in the 3 phases commonly reported by sufferers of the disease:

1. Premonitory symptoms;

Some patients are able to predict the onset of an migraineous episode due to the presence of premonitory symptoms that occur up to 48 hours before an attack (Giffin *et al.*, 2003). These may include changes in mood such as irritability, depression, euphoria, increased food cravings for sweet foods or conversely anorexia, changes in motivation such as excessive sleepiness and difficulty in concentrating and in many subjects, repetitive yawning. Additionally some patients (less than a third) also report aura type symptoms.

2. The migrainous headache;

This is often the greatest source of disability in many patients and often occurs with associated nausea and sensitivity to external stimuli. In about two thirds of patients the headache is unilateral and bilateral in the remainder (Selby & Lance, 1960). The quality of the pain is often pulsating and largely involves the frontotemporal region. Pain is also commonly referred to the facial skin

3. "Postdrome";

Resolution of the headache often leaves sufferers feeling exhausted for the following 24 hours and many experience similar symptoms to those of premonitory symptoms.

The separation of a migraine attack in to different phases along with the absence of migrainous symptoms between attacks is suggestive of a complex pathology. As attacks occur regularly and can sometimes be associated with premonitory symptoms

such as alterations in appetite, wakefulness and mood, a role of the hypothalamus and a migraineous "brain" seems likely. However, some migraine attacks appear to be triggered in response to afferent stimulation such as flickering light, noise and some smells, whereas other migraine triggers, such as nitrates, appear to act primarily on cranial blood vessels. The potential role that higher brain centres, cranial blood vessels and a genetic susceptibility may play in initiating a migraine episode is discussed further in sections 1.3.5, 1.5 and 1.9.

There is considerable debate concerning whether the different forms of migraine, in particular migraine without aura and migraine with aura, are distinct clinical entities or whether the underlying pathology of both disorders is shared. Similarly, many clinicians think of tension-type headache and migraine as related diseases occupying different ends of a spectrum. In support of this, many patients tend to suffer from more than one type of primary headache syndrome.

The term cervicgenic headache was initially suggested by Sjaastad and colleagues (Fredrikson *et al.,* 1987; Sjaasatd *et al.*, 1990) and described episodic unilateral headaches lasting from 3 hours to a week and reoccurring at intervals from 2 days to 2 months, considered to arise from the cervical spine. As with migraine, these headaches more frequently occurred in women than men and were accompanied by nausea, vomiting and photophobia, however unlike migraine they could be precipitated by neck movement. Headache patients often report pain that involves the front of the head, innervated by the ophthalmic division of the trigeminal nerve but also pain at the

back of the head, resulting from the innervation of the greater occipital nerve (Anthony, 1992). This overlap of pain is suggestive of a convergence of afferent input from the meninges and upper cervical roots and it has now been shown in rat that stimulation of the greater occipital nerve induces increased central excitability of dural afferents (Bartsch & Goadsby, 2002, 2003). Hence, while the focus of this study is the head pain occurring during migraine without aura, due to the overlap of pathways postulated to be involved in different primary headache syndromes, many of the concepts discussed may also have relevance to the pathology of other headache types.

1.2.1 Migraine Prevalence

The prevalence of migraine (i.e. the proportion of a population affected over a defined period) has been extensively studied with considerable variation reported between populations. This variation in prevalence was examined following meta-analysis of 18 published population-based studies (Scher *et al.,* 1999), who found that when gender differences were not considered, age and geographical location were responsible for much of the variance seen between studies. However, methodological factors such as sampling method, response method and response rate also impacted on reported variance.

Country	Method	Sample size	Migraine prevalence (%)		Reference
			Females	Males	
USA	Posted questionnaire	20,468	17.6	5.7	Stewart *et al.*, 1992
UK	Clinical interview	16,002	11.0	4.3	Cull *et al.*, 1992
Switzerland	Clinical interview	379	32.6	16.1	Merikangas *et al.*, 1993
Hong Kong	Telephone	7,356	1.5	0.6	Wong *et al.*, 1995
Saudi Arabia	Clinical interview	22,630	6.8	3.2	Al-Rajeh *et al.*, 1997
Danish	Posted questionnaire	5360	19% migraine without aura 8% migraine with aura	7% migraine without aura 7% migraine with aura	Russell *et al.*, 2002

Table 2 Summary of some of the studies examining migraine prevalence in different geographical populations

Despite the variance in these studies, two main factors with regards to migraine prevalence are obvious. Firstly, the greater incidence of migraine in females provides researchers with an immediate insight to the possible role of female hormones in generating susceptibility to attacks. However, episodic and chronic tension type headache prevalence also show a world wide higher incidence in females than males,

although the gender difference is not as considerable as for migraine (for review see Lipton *et al.*, 2001). In the second case, migraine is considerably more prevalent in Europe and America than in Asia and Africa, however possible reasons for this are much more complex and encompass a whole range of factors such as diet, stress, genetic and environmental influences as well as a possible cultural component.

1.2.2 Social impact of migraine

The direct costs associated with migraine such as physician consultation, prescriptions and laboratory procedures are relatively low in comparison with many illnesses, probably due to the low rate of hospitalisation among sufferers. The indirect costs though, are substantial and largely due to sickness related absenteeism and reduced productivity at work. However, the economic burden of the migraine is only one effect on society that this disease exerts. For the migraine sufferer, coping with a frequently occurring debilitating disease that influences mood, is likely to put great deal of pressure on family dynamics, especially as migraine incidence peaks during childbearing years, when sickness is likely to cause maximum disruption. In an attempt to validate the impact of migraine on patient's lives, the use of the Headache Impact Test (HIT) and the Migraine Disability Assessment (MIDAS) and being increasingly recommended in providing individual coping strategies (for review see Dowson, 2001).

1.3 Anatomy of the trigeminovascular system

Despite the many theories of migraine pathophysiology, researchers agree that the trigeminovascular system, which encompasses the large intracranial arteries, dura

mater and their neuronal innervation, plays a fundamental role in some manifestations of the disease. An understanding of the interconnections between these structures is hence crucial in understanding the mechanism of head pain and as such, the components of the trigeminovascular system are reviewed below.

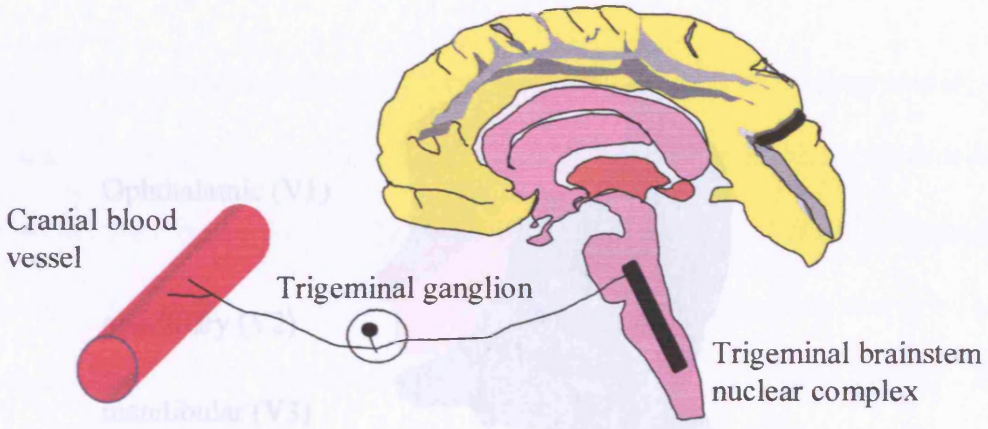


Figure 1. The Trigeminovascular system

The key components of the trigeminovascular system encompass the pain producing cranial vessels and dura mater, the peripheral branches of the trigeminal nerve innervating the cranial vessels and dura mater and the central terminations of the trigeminal nerve which synapse in the trigeminal brainstem nuclear complex.

1.3.1 The Trigeminal nerve

The trigeminal or fifth cranial nerve has motor (mastication, middle ear and throat muscles), proprioceptive (temporomandibular joint) and other sensory functions. Trigeminal means "three twins" and as such, the sensory distribution of the trigeminal nerve comprises three divisions, all arising from the trigeminal ganglion; the

ophthalmic (V_1), the maxillary (V_2) and the mandibular (V_3) divisions. The trigeminal nerve is the only cranial nerve to be involved in sensory cutaneous innervation and provides the sole sensory innervation of facial skin and most of the innervation of the head, although some parts of the head also receive innervation from the upper cervical spinal nerves or other cranial nerves (Sessle, 2002).

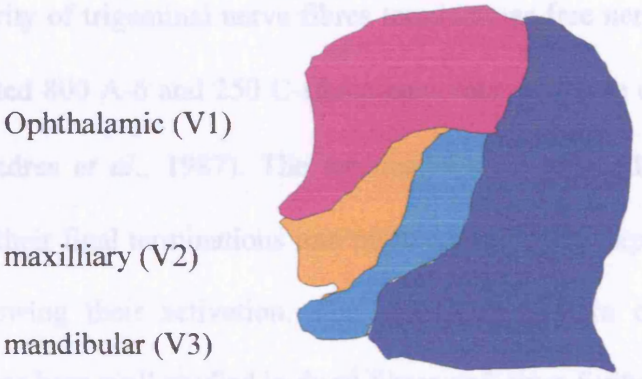


Figure 2. The innervation territories of the trigeminal nerve

The sensory innervation of the face occurs through the three divisions of the trigeminal nerve; the ophthalmic (V_1), the maxillary (V_2) and the mandibular (V_3) divisions.

The terminations of trigeminal afferent fibres can be of several types. Some end in association with sensory organs and respond to light tactile stimuli, known as low threshold (LT) mechanoreceptors. Some respond to muscle stress and tension, the proprioceptors. Others, the nociceptors, terminate as free nerve endings and are activated by painful noxious stimulation. Nociceptors, under physiological conditions, are associated with two kinds of primary afferent fibres, the first are slow-conducting, small diameter, myelinated A-δ fibres and the second type, are even slower

conducting, smaller diameter, unmyelinated C-fibres. These afferent fibres project into the central nervous system (CNS), where their first point of synapse occurs in either the trigeminal brainstem nuclear complex (VBNC) or upper cervical dorsal horn.

1.3.2 Morphology of nerve fibres innervating the dura mater

1.3.2.1 Sensory innervation

The majority of trigeminal nerve fibres terminate as free nerve endings and in the rat an estimated 800 A-δ and 250 C-fibres innervate each side of the supratentorial dura mater (Andres *et al.*, 1987). The terminations of A-δ and C-fibres are commonly mixed in their final terminations into multi-axonal units, explaining the varied effects seen following their activation. The expression pattern of neurotransmitters and peptides has been well-studied in dural fibres and gives further information about their functional properties. Calcitonin gene related peptide (CGRP) and Substance P (SP) immunoreactivity are both extremely prominent along the meningeal arteries and venous sinuses and also show a strong association with dural mast cells (Dimitriadou *et al.*, 1991). (For a comprehensive review of the projections of trigeminal afferents to the dura mater see Moskowitz *et a*l., 1987).

1.3.2.2 Autonomic innervation of dural and intracranial vessels

The major innervation of the tentorium, falx and venous sinus, occurs from the tentorial nerve, which arises from the cavernous plexus, which in turn, has been shown in the monkey, to be formed from fibres of the ophthalmic division of the trigeminal nerve and sympathetic fibres from the superior cervical ganglion (Ruskell, 1988).

Perivascular sympathetic fibres accompany the middle meningeal artery (MMA) and build up a dense plexus around the sagittal sinus (Cavaollotti *et al.*, 1998). Anteriorly, parasympathetic fibres from the pterygopalatine (sphenopalatine) ganglion, and fibres from the maxillary division of the trigeminal nerve join the cavernous plexus and are distributed along the middle cerebral artery (Cavanagh *et al.*, 1990).

1.3.3 Primary afferent cell bodies in the trigeminal system

The cell bodies of trigeminal primary afferent neurons are located in the trigeminal ganglion in the peripheral nervous system. The presence of many classical peptide transmitters, such as glutamate, GABA, glycine, serotonin, noradrenaline, acetylcholine, cholecystokinin, somatostatin and opioids peptides, as well as neuroactive molecules like nitric oxide have been demonstrated within the trigeminal ganglion (for review see Lazarov, 2002). The co-existence of neuropeptides, such as SP and CGRP and transmitters in a single neuron is also evident. The discovery of such chemical heterogeneity has influenced the view that the trigeminal ganglion is at the centre of a complex plastic system that is highly susceptible to the environment of its primary afferent terminations (for review see Lazarov, 2002). However, there are currently no studies that have explored whether the activity of the trigeminal ganglion is altered in migraineurs, although recently, the technique of functional magnetic resonance imaging has been suggested as a means of such an investigation (Borsook *et al.*, 2004).

1.3.4 The Dura mater

The dura mater is the most superficial layer of the three connective tissue layers, the meninges, surrounding and protecting the brain and spinal cord. The dura mater surrounding the brain is tightly attached to and continuous with the periosteum of the cranial vault. Three dural folds extend into the main brain fissures to form the dural sinuses which collect most of the blood returning from the cranial circulation via the vein of Galen, the emissary and dipole vein. The dura mater itself, is richly supplied by meningeal arteries, veins and an extensive capillary network, especially in the parasagittal region (Kerber & Newton, 1973), which apart from a nutritive function, are thought to be involved in the regulation of intracranial pressure (Lang *et al.*, 1971).

1.3.5 The cerebral vasculature

The pivotal studies of Ray and Wolff (1940) on the perception of pain in conscious patients following stimulation of discrete regions of the dura mater identified the proximal regions of cerebral and dural arteries along with the large venous sinuses as important structures in the recognition of pain. Following the realisation, that stimulation of cranial vessels could evoke painful responses, much effort has been devoted to studying cranial blood flow abnormalities during migraine as a potential source of the pain (Martins *et al.*, 1993; Nichols *et al.*, 1990).

Stimulation site	Area of pain perception
Middle meningeal artery distension	back of the eye
Middle cerebral artery	
-proximal section	eye, forehead & temple
- middle section	retro-orbital pain
-distal section	above the eye
Intracranial segment of internal carotid artery	lateral to the eye
Vertebral artery	occiput
Anterior cerebral arteries	eye, forehead & temple
Superior sagittal sinus	frontoparietal area

Table 3. Main cranial structures resulting in pain perception as identified by Ray and Wolff (1940)

1.3.5.1 Intracranial vessels

Reduced regional blood flow has been observed using transcranial Doppler sonography in the middle cerebral artery (MCA) on the headache side, during migraine attacks (Friberg *et al.*, 1991), which is assumed to result from vasodilatation. In this study, following anti-migraine treatment, the headache was aborted and blood flow returned to normal, although the timing of the relief and the flow change were not related. These observations indicated that dilatation is at the very least, a reactive response during migraine in some patients, it does not necessarily follow however, that distended intracranial vessels are the primary source of pain during migraine. Further anecdotal evidence for abnormality of the intracranial arteries in migraine is the aggravation of symptoms by coughing, jolting or breath holding.

1.3.5.2 Extracranial arteries

The innervation of extracranial arteries is separate from that of intracranial arteries with convergence of afferent fibres occurring only in the trigeminal brainstem nuclear complex (VBNC) (Borges & Moskowitz, 1983). The concept of abnormal extracranial artery dilatation during migraine is supported by the observations in some subjects that the throbbing nature of the headache appears to be synchronous with pulsations of the temporal artery. Indeed Blau and Dexter (1981) showed that the inflation of a sphygmomanometer cuff around a patient's head could relieve headache symptoms in about one third of those tested. However, the number of patients responding to compression of the temporal artery is small and many migraine patients do not even experience a throbbing component of their head pain. Hence, it is likely that extracranial vessel distension contributes to migraine pain in only a minority of subjects.

1.3.6 Trigeminal brainstem nuclear complex

The VBNC is a bilateral, multinucleated structure extending rostrally from the pons and caudally to the upper cervical spinal cord. Its main sub-divisions include the principal or main sensory nucleus (Vp) and the spinal trigeminal nucleus (Vsp) which consists of three subnuclei, namely the oralis (Vo), interpolaris (Vi) and caudalis (Vc). Unlike the organisation of the rest of the spinal cord, trigeminal collaterals ascend and descend in a trigeminal spinal tract, which runs throughout the VBNC, creating a convergent system (Lovick & Wolstencroft, 1983). In addition to spinal trigeminal tract neurons, lateral to each nucleus, groups or "islands" of neuronal elements known as the paratrigeminal nucleus are also apparent, which further facilitate transmission of neuronal signals throughout the VBNC.

The structural organisation of the VBNC, with the exception of Vc is fairly uniform, with a somatotopic arrangement. The ventral part of each subnuclei or nuclei generally receives input from primary afferents from the V_1 division, the dorsal part, from V_3 and the part in between from V_2. An especially highly organised feature of the VBNC is vibrissal representation which runs in rostrocaudal columns and is conserved both in the thalamus and somatosensory cortex, indicating the importance of these structures in the exploration of the external environment.

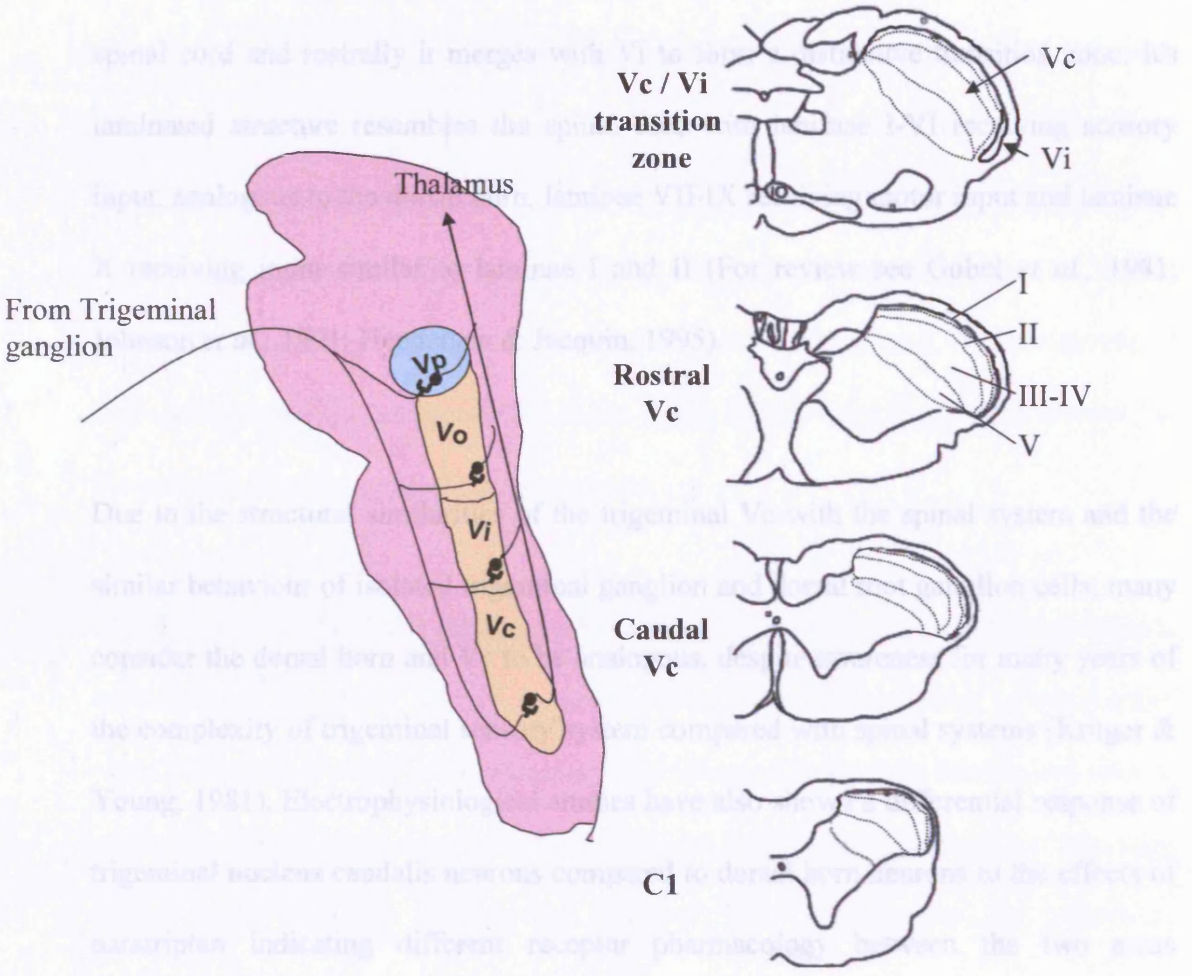


Figure 3. The Trigeminal Brainstem Nuclear Complex (VBNC)

The VBNC contains the principal sensory nucleus (Vp) and the spinal trigeminal nucleus (Vsp) which consists of three subnuclei, namely the oralis (Vo), interpolaris (Vi) and caudalis (Vc). Like the spinal dorsal horn, the Sp5C has a laminated structure and contains in laminae I and V numerous nociceptive neurones, many of which project directly to higher brain centres involved in pain processing

The Vc is the largest component of Vsp, at its caudal extremity, it merges with the spinal cord and rostrally it merges with Vi to form a distinctive transition zone. It's laminated structure resembles the spinal cord with laminae I-VI receiving sensory input, analogous to the dorsal horn, laminae VII-IX receiving motor input and laminae X receiving input similar to laminae I and II (For review see Gobel *et al.,* 1981; Johnson *et al.,* 1991; Henderson & Jacquin, 1995).

Due to the structural similarities of the trigeminal Vc with the spinal system and the similar behaviour of isolated trigeminal ganglion and dorsal root ganglion cells, many consider the dorsal horn and Vc to be analogous, despite awareness for many years of the complexity of trigeminal sensory system compared with spinal systems (Kruger & Young, 1981). Electrophysiological studies have also shown a differential response of trigeminal nucleus caudalis neurons compared to dorsal horn neurons to the effects of naratriptan indicating different receptor pharmacology between the two areas (Cumberbatch *et al.,* 1998). Analysis of the type of input the Vc receives in comparison to the dorsal horn has been gained from exploration of the presence of the neuropeptide nociceptive markers, CGRP and SP and the expression of the cell surface marker isolectin I-B4 (IB4) (Snider & McMahon, 1998). CGRP and SP-positive fibres project mainly to laminae I and IIo of the dorsal horn, which shows little IB4 immunoreactivity, where as in Vc, a significant immunoreactivity is seen for both fibre types across I, IIo and Iii as well as I-B4 (Sugimoto *et al.,* 1997).

The Vi /Vc transition zone however tends to resemble the dorsal horn, indicating that the caudal Vc and Vi /Vc border receive a different compliment of afferent

nociceptive input (Bereiter *et al.*, 2000). An elegant study by Meng and colleagues showed that electrical corneal stimulation, activated cells in both the Vi /Vc transition zone and Vc/ C_1 border, however the cell populations (based on receptive field properties, activation by algesic substances and their supraspinal projections) were significantly different (Meng *et al.*, 1997),. The authors suggested that the Vc /C_1 population was more likely to be involved in motor reflexes and the recruitment of descending pathways, while neurons at the Vi /Vc border were likely to be involved in the conscious perception and reaction to pain.

While several studies have focused on the characterisation of the Vc, relatively little is known about the role of Vo and even less about Vi in the processing of nociceptive pain. Lesioning of these areas suggests a significant disruption to the transmission of craniofacial pain, hence electrophysiological and immunocytochemical investigations would prove useful in relating the different VBNC components to behaviour, autonomic and motor effects seen clinically.

In the spinal cord, afferent nerves project to a single segment, where as the VBNC has a unique feature of dual representation, where afferents projecting from the same craniofacial structures synapse in different areas of the VBNC. At present, there is only evidence for this phenomenon in structures innervated by V_1 and the functional relevance is not clear. It may facilitate different responses to nociceptive stimuli by projection to different supraspinal structures or purely represent a redundant pathway.

1.3.6.1 Characterisation of second order neurons in the trigeminal brainstem nuclear complex

The result of numerous electrophysiological studies on the response of second order neurons within the spinal cord and VBNC has led to a widely accepted classification into three basic classes of sensory neuron (Sessle, 2000):

i.) Nociceptive specific (NS); responding only to noxious stimulation (e.g. pinch) of the receptive field and activated by A-δ and C-fibres.

ii.) Wide dynamic range (WDR); responding with a graded intensity to both tactile and noxious stimulation, activated by A-β, A-δ and C-fibres.

iii.) Low threshold (LT); responding only to tactile stimuli and activated by A-β primary afferents only.

NS and WDR cells are typically located in the superficial (I / II) and deep laminae (V / VI) of Vc. The NS cells tend to have a small receptive field, where as the receptive fields of WDR cells are much larger. This classification applies to cells throughout VBNC (for review see Dunber *et al.*, 1978), although examination of cells in the rostral VBNC is limited, with only a few studies of neuronal properties carried out on Vo cells (Dallel *et al.*, 1990, 1999). The studies in this thesis, largely involve examination of WDR cells in the VBNC, activated by noxious electrical stimulation of the dura. In most cases, these second-order neurons could also be activated by stimulation of the facial receptive field (FRF).

Electrical stimulation of the dura mater is assumed to be nociceptive in character as the dura mater is almost exclusively innervated by A-δ and C-fibres (Andres *et al.*, 1987). This is further supported by studies showing that dural stimulation results in *c-fos* expression in the Vc, which has been previously shown to be well correlated to the magnitude of nociceptive insult (Munglani & Hunt, 1995).

1.4 Transmitters in the Trigeminovascular system

Immunocytochemical studies have reported a wide variety of neurochemicals or their cellular receptors in the VBNC, all of these may play a significant role in the modulation of VBNC activity. However, headache research in the last decade has very largely focused on CGRP, SP and 5-HT for their possible involvement in migraine.

1.4.1 Calcitonin gene related peptide (CGRP)

Calcitonin gene related peptide (CGRP) is the most abundant peptide transmitter found in perivascular sensory trigeminal nerve fibres, where it is colocalized with substance P and other neurotransmitters (Uddman *et al.*, 1985). Evidence for a role of CGRP in the pathophysiology of migraine, comes from the observation that the levels of CGRP in blood from the external jugular vein, which drains blood from extracranial tissue including the dura and the trigeminal ganglion, is increased during spontaneous migraine attacks (Goadsby *et al.*, 1990). Furthermore, this increased CGRP level is normalised following administration of the anti-migraine treatment, sumatriptan (Goadsby *et al.*, 1993).

A recent study provided evidence that as well as an involvement in the pain phase of migraine, CGRP may also play a role in the generation of attacks. In this study, CGRP infusion into patients suffering from episodic migraine, outside of an attack resulted in an immediate headache during the infusion in all subjects studied. Furthermore, all subjects developed a delayed headache approximately 5 hours after the infusion, which in 3 out of 9 subjects was of sufficient magnitude to fulfil the IHS diagnostic criteria of migraine (Lassen *et al.*, 2002).

In animals, stimulation of trigeminal sensory nerve fibres causes antidromic release of CGRP with subsequent vasodilatation of the cerebral vasculature (Beattie & Connor, 1994; Escott *et al.*, 1995). Furthermore, CGRP infusion in addition to generating dilatation of the MMA also increased firing of Vc neurons (Cumberbatch *et al.*, 1999). However, this increase in Vc neuronal activity was short lived (approximately 6 minutes) and hence unlikely to be directly responsible for long term sensitisation, however, identification of the downstream processing following CGRP infusion is likely to further understanding of the molecular processes involved in the generation of headache. Indeed, a causative role of CGRP has now been confirmed following the recently described efficacy of CGRP antagonists in the treatment of acute migraine (Brain *et al.*, 2002; Doods *et al.*, 2000; Olesen *et al.*, 2004).

1.4.2 Substance P

SP is a potent neurochemical involved in the transmission of pain signalling and is released from both peripheral and central terminals of primary afferent fibres following nociceptive stimuli in both the spinal and trigeminal systems (for review see

Harrison & Geppetti, 2001). The co-localisation of SP with CGRP in the trigeminal system and its nociceptive character, led to exploration of SP antagonists as potential anti-migraine drugs. Pre-clinically, SP antagonists are highly potent in blocking both plasma protein extravasation in a model of neurogenic inflammation (Cutrer *et al.*, 1995; O'Shaughnessy *et al.*, 1994) and *c-fos* expression in the VBNC following trigeminal ganglion and meningeal stimulation (Clayton *et al.*, 1997; Cutrer *et al.*, 1995; Shepheard *et al.*, 1993). However, they have no effect on Vc firing elicited by superior sagittal sinus (SSS) stimulation in the anaesthetised cat (Goadsby *et al.*, 1998).

Clinically, SP antagonists have been reported to have no significant effect over placebo either in the acute (Connor *et al.*, 1998; Diener, 2003) or prophylactic (Goldstein *et al.*, 1997) treatment of migraine, indicating that the blockade of neurogenic inflammation, at least in the species tested, is not sufficient in predicting anti-migraine activity of compounds.

1.4.3 Serotonin

Serotonin or 5-hydroxytriptamine (5-HT) is widely distributed throughout the body and in particular in the gastrointestinal tract, platelets and the brain with seven major classes of receptor ($5\text{-HT}_{1\text{-}7}$) and further subclasses in each division. There is considerable circumstantial evidence, which suggests the involvement of serotonin (5-HT) in the pathophysiology of migraine (for a detailed review see Silberstein *et al.*, 2001). Decreases in platelet 5-HT concentration, increase in urinary 5-HT and increase in 5-HIAA, the major metabolite of 5-HT (Ferrari *et al.*, 1989) have been reported during attacks. Furthermore, headache can be aborted by 5-HT_1 agonists (Humphrey

& Feniuk, 1991; Silberstein, 1994) and induced such as reserpine, which causes an increase in endogenous 5-HT concentrations, an effect that can be prevented by pre-treatment with methysergide, a 5-HT_2 antagonist. Centrally penetrating 5-HT_1 agonists such as naratriptan and zolmitriptan, also inhibit the activity of Vc neurons responding to noxious dural stimulation (Goadsby & Hoskin, 1996) as well as constricting meningeal, dural and pial blood vessels (Ferrari & Saxena, 1993; Longmore et al., 1997; Humphrey & Feniuk, 1991) and this is discussed further in section 1.8.3.2.

1.4.4 Other transmitters modulating VBNC activity

GABA is an inhibitory neurotransmitter released from local interneurons within the substantia gelatinosa of the VBNC. Agonists acting both through the $GABA_A$ and $GABA_B$ receptor subtype appear to inhibit Vc neurons following noxious electrical stimulation of the SSS (Storer *et al.*, 2001). This GABA mediated inhibition appears dependant on C-fibre activation, as neonatal capsaicin disrupts the modulation (Chiang *et al.*, 1999). Exogenous opioids are also capable of decreasing Vc neuronal activity when administrated directly to the VBNC or into the supraspinal structures influencing the activity of the VBNC, indicating wide spread distribution of opiate receptors in the trigeminal system. There is also significant evidence to support a role of nitric oxide in modulation of the trigeminovascular system and this is discussed in detail in Chapter 3.

1.5 Modulation of neuronal processing in the Trigeminal brainstem nuclear complex

As discussed in section 1.2, migraine is not only characterised by headache that has a component of referred pain in the head and face, but also by distinct behavioural changes (e.g. loss of appetite, mood changes). The presence of premonitory symptoms that allows some migraineurs to predict the onset of an attack (Giffin *et al.*, 2003; Jacome, 2001; Santoro *et al.*, 1990) and the susceptibility to "ice cream-headache" that migraineurs exhibit (Drummond & Lance, 1984) indicates the involvement of central brain regions in the generation of an attack. While the studies in this thesis have not directly examined the activity of these higher centre structures, it is of relevance to discuss the ascending and descending connections with the VBNC as well as the control systems within the VBNC, for an understanding of how the responses of the trigeminovascular system may be altered during migraine.

1.5.1 Neuronal modulation within the Trigeminal brainstem nuclear complex

The gate control theory of pain was first proposed by Wall & Melzack in the 1960's and suggested that the relay of pain signalling between the primary afferent neurons entering the dorsal horn and VBNC could be significantly modulated before transmission to the brain (Wall & Melzack, 1994). The theory suggests that following activation of fast conducting primary afferents, the dorsal horn becomes a "locked gate" and prevents further afferent input synapsing with ascending pathways. This theory is now widely accepted, although the precise contribution of these inhibitory circuits to specific pathways is unknown.

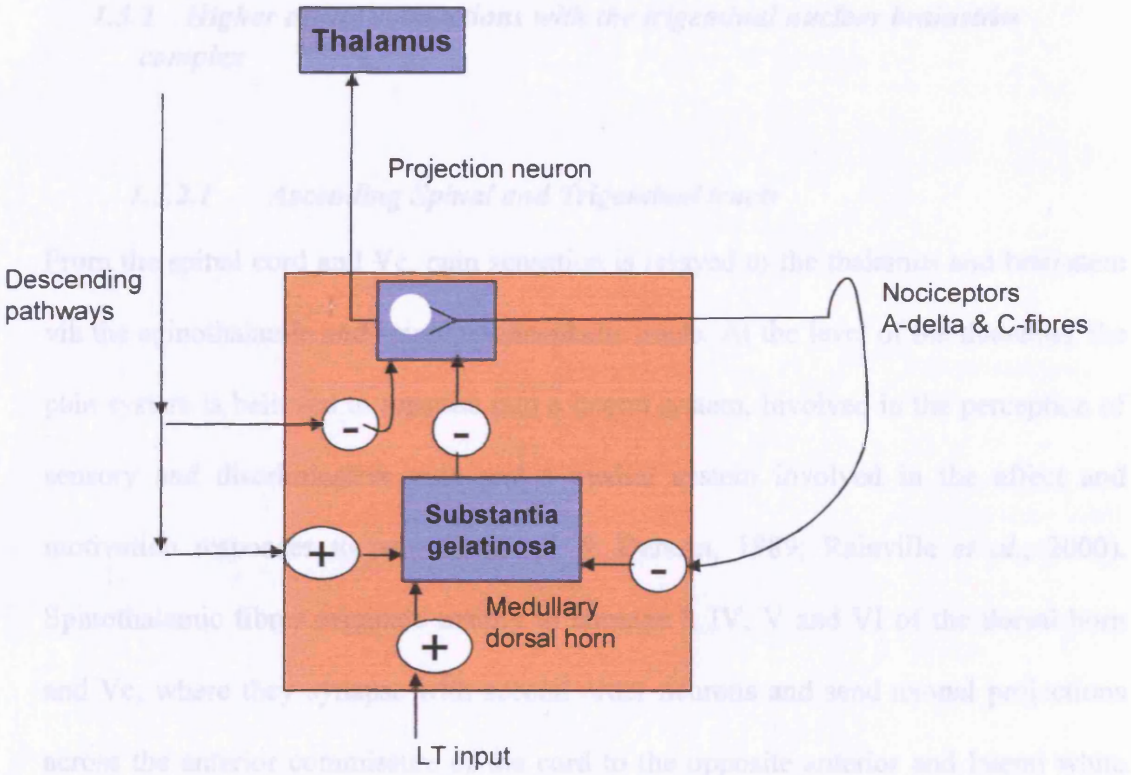


Figure 4. Schematic representation of "gate control"

The gate control hypothesis suggests that the pathway of impulses from the primary afferent neurons to the thalamus are regulated by inhibitory interneurons originating in the substantia gelatinosa of the dorsal horn. The interneurons are activated by descending inhibitory neurons or non-nociceptive afferent input and inhibited by C-fibre input.

1.5.2 Higher centre connections with the trigeminal nuclear brainstem complex

1.5.2.1 Ascending Spinal and Trigeminal tracts

From the spinal cord and Vc, pain sensation is relayed to the thalamus and brainstem via the spinothalamic and spinomesencephalic tracts. At the level of the thalamus, the pain system is believed to separate into a lateral system, involved in the perception of sensory and discriminative pain and a medial system involved in the affect and motivation responses to pain (Bushnell & Duncan, 1989; Rainville *et al.*, 2000). Spinothalamic fibres originate mainly in laminae I, IV, V and VI of the dorsal horn and Vc, where they synapse with second order neurons and send axonal projections across the anterior commissure of the cord to the opposite anterior and lateral white columns These fibres then ascend to the brain in both the anterior spinothalamic tract and lateral spinothalamic tract. The anterior spinothalamic tract is located in the anterior funiculus of the spinal cord and is intermingled with ascending spinoreticular and spinomesencephalic tract fibres. Laterally the tracts are continuous with the lateral spinothalamic tract, located in the lateral funiculus. At the level of the lower brainstem, spinothalamic tract axons separate and those of the anterior tract join the medial lemniscus, while the axons of the lateral tract continue as the spinal lemniscus, with both lemnisci ascending to the thalamus. Specific ascending tracts from the trigeminal system are shown in the figure 5 and are essentially synonymous with those from the spinal system, with axons from the spinal trigeminal nucleus, joining the spinothalamic, spinomesencephalic and spinoreticular tracts to form the trigeminothalamic tract (Grays anatomy, 1995).

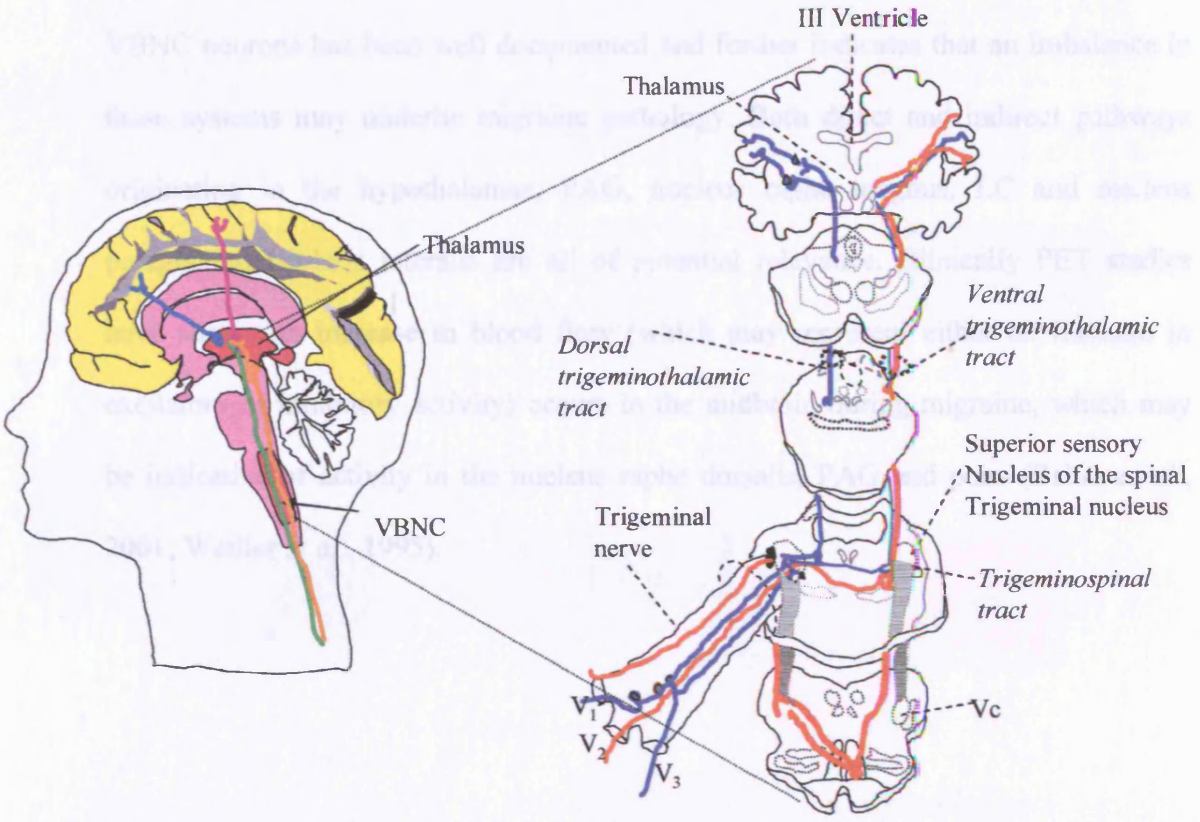


Figure 5. Ascending spinal trigeminal tracts conveying pain information

The spinothalamic tract (green) projects to the somatosensory nucleus of the thalamus, which in turn projects to the somatosensory cortex for the perception of pain. The spinothalamic tract also projects to other thalamic nuclei, which in turn are thought to project to the anterior cingulated cortex to modulate the emotional aspects of pain (blue). Spinomesencephalic tract neurons (orange) ascend in association with the spinothalamic tract and project to various midbrain structures, where they are thought to be involved in the motivational-affective component of pain. Pain, thermal and tactile fibres, originating from the spinal trigeminal nucleus (red), cross at various locations in the lower brainstem and ascend in the ventral trigeminothalamic tract in association with the contralateral medial lemniscus. Fibres conveying touch and pressure (blue) ascend by two separate pathways; 1.) Fibres from the ventral portion of the spinal trigeminal nucleus cross and ascend with the contralateral medial lemniscus. 2.) Fibres from the dorsomedial part of the spinal trigeminal nucleus ascend, uncrossed as the dorsal trigeminal tract. Both dorsal and ventral trigeminal tracts project to the ventral posteromedial nucleus of the thalamus. (Adapted from Grays Anatomy, 1995).

1.5.2.2 Descending Spinal and Trigeminal tracts

Pre-clinically the involvement of descending pathways in modulating the activity of VBNC neurons has been well documented and further indicates that an imbalance in these systems may underlie migraine pathology. Both direct and indirect pathways originating in the hypothalamus, PAG, nucleus raphe magnus, LC and nucleus paragigantocellularis lateralis are all of potential relevance. Clinically PET studies have shown an increase in blood flow (which may represent either an increase in excitatory or inhibitory activity) occurs in the midbrain during migraine, which may be indicative of activity in the nucleus raphe dorsalis, PAG and pons (Bahra *et al.*, 2001; Weiller *et al.*, 1995).

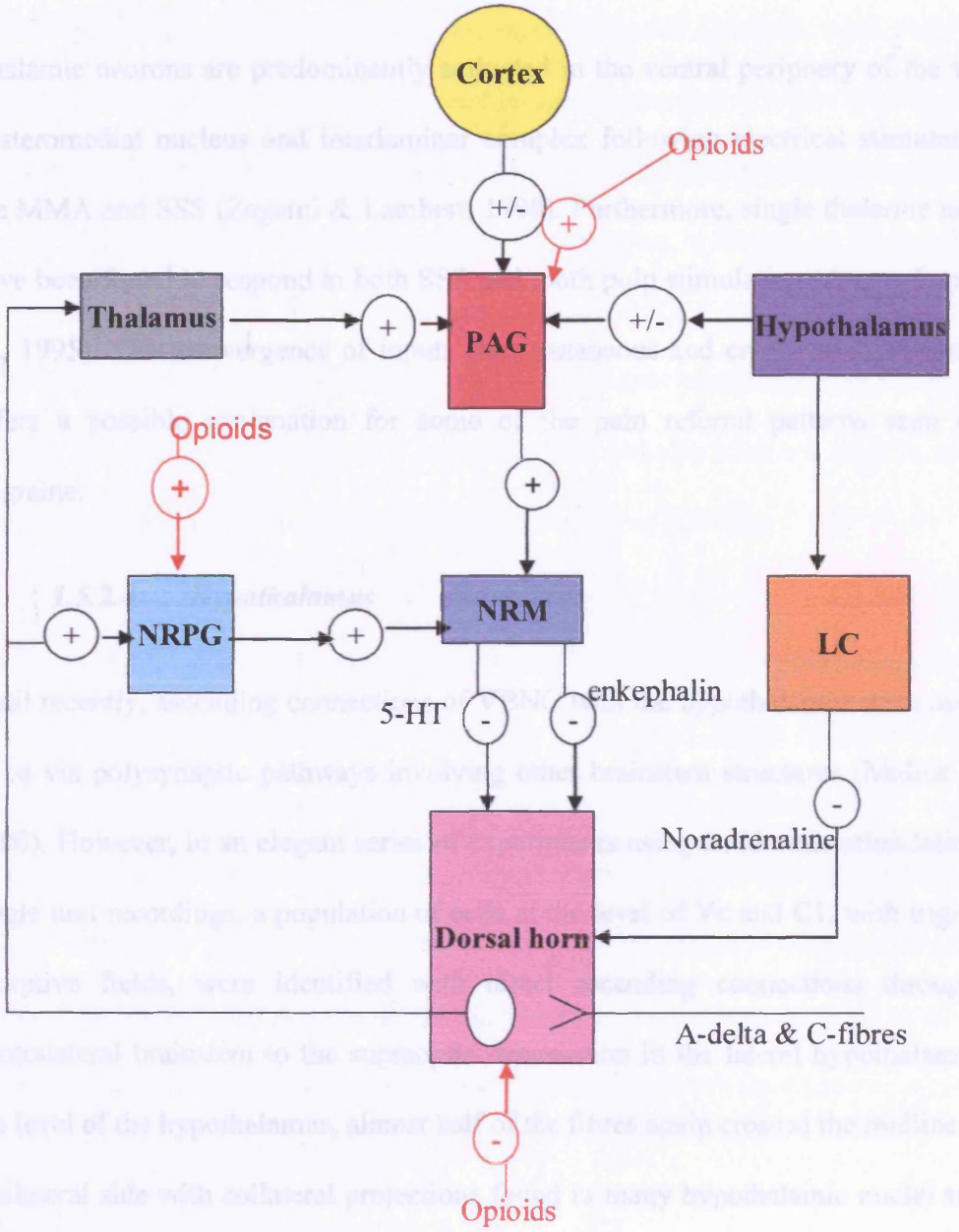


Figure 6 Analgesia system of the brain & spinal cord

The above schematic diagram is an extremely simplified version of how descending pathways act to dampen the activity in the dorsal horn and Vc. For example, opioids may excite neurons in the PAG, which in turn activates neurons in the NRM, which result in activation of inhibitory 5-HT and enkephalin-containing neurons, which project to the substantia gelatinosa. (Diagram adapted from Rang et al., 1995). PAG, periaqueductal grey: LC, locus ceruleus: NRM, nucleus raphe magnus: NRPG, nucleus reticularis paracellularis.

1.5.2.3 Thalamus

Thalamic neurons are predominantly activated in the ventral periphery of the ventral posteromedial nucleus and interlaminar complex following electrical stimulation of the MMA and SSS (Zagami & Lambert, 1990). Furthermore, single thalamic neurons have been found to respond to both SSS and tooth pulp stimulation (Angus-Leppan *et al.*, 1995). This convergence of inputs from cutaneous and craniovascular structures offers a possible explanation for some of the pain referral patterns seen during migraine.

1.5.2.4 Hypothalamus

Until recently, ascending connections of VBNC with the hypothalamus were assumed to be via polysynaptic pathways involving other brainstem structures (Malick *et al.*, 2000). However, in an elegant series of experiments using antidromic stimulation and single unit recordings, a population of cells at the level of Vc and C1, with trigeminal receptive fields, were identified with direct ascending connections through the contralateral brainstem to the supraoptic decussation in the lateral hypothalamus. At the level of the hypothalamus, almost half of the fibres again crossed the midline to the ipsilateral side with collateral projections found in many hypothalamic nuclei such as suprachiasmatic and supraoptic nuclei and structures rostral to the hypothalamus such as the caudate-putamen, globus pallidus and substantia innominata.

The functional relevance of these direct VBNC-hypothalamic connections merit further investigation, for example, the current understanding of the function of the suprachiasmatic nucleus, makes it ideally placed for an involvement in migraine. As

well as showing sexual dimorphism (in females it is elongated, whereas in males it is spherical in shape), it is considered to be important in the control of circadian rhythm in humans and neuronal activity within it is directly regulated by photic stimuli. The suprachiasmatic nucleus is also thought to be involved in regulating the serotonin system and melatonin concentrations, which are known to be altered in migraineurs e.g. significant seasonal variations in hypothalamic 5-HT content, cerbro-spinal fluid 5-HIAA, platelet 5-HT uptake and platelet 5-HT content have been found in migraine patients (Ferrari *et al.*, 1993) and a low melatonin concentration has been reported in both episodic and chronic migraine sufferers (Peres *et al.*, 2001).

Activation of the preoptic nucleus results in profound inhibition of the activity of the Vc. This effect is mediated, at least in part, by pathways directed via the ventrolateral PAG, the nucleus raphe magnus or the nucleus paragigantocellularis, since lesions in these pathways reduce hypothalamic mediated inhibition of Vc activity (Mokha *et al.*, 1987). The extent to which inhibition of Vc activity is mediated via projections from the hypothalamus to the nucleus raphe magnus or the nucleus paragigantocellularis, or via projections routed through the periaqueductal gray matter is not known.

1.5.2.5 Periaqueductal grey

The periaqueductal grey (PAG) is the grey matter surrounding the midbrain aqueduct. Its anatomical organisation involves functional neuronal columns, the dorsolateral, dorsomedial, lateral and ventrolateral columns of which, the lateral and ventrolateral columns receive a dense afferent projection from Vc and Vo (Wiberg *et al.*, 1986). Activation of these ascending trigeminal projections results in a wide range of

behavioural (Fields & Basbaum, 1999) and cardiovascular responses (Carrive & Bandler, 1991). There is also significant evidence that stimulation of the periaqueductal grey (PAG) can produce migraine like headaches in humans (Raskin *et al.*, 1987; Veloso *et al.*, 1998). A role of the PAG in the generation of migraine is supported by a recent clinical communication regarding a patient with a cavernoma in the midbrain that bled. The patient previously had no history of headache and now suffers from frequent migraine (Goadsby, 2002).

There are a considerable number of direct projections from the PAG to the VBNC (Blomqvist & Craig, 1991) as well as indirect connections via the NRM. The ability of the PAG to modulate VBNC activity has been determined in animal models where stimulation of the ventrolateral PAG produces analgesia and reduces neuronal activity (Levine *et al.*, 1991; Knight & Goadsby, 2001). A dysfunction of PAG descending pathways has been suggested to underlie neuronal hypersensitivity of the VBNC that may occur during a migraine attack (Knight & Goadsby, 2001; Knight *et al.*, 2002). In support of the theory of the PAG as a source of abnormality in headache, are the results of a clinical study examining iron homeostasis, thought to be representative of cellular function in both episodic migraine and chronic daily headache patients. In both groups of patients, iron homeostasis was found to be selectively impaired in the PAG, indicating the possible role of the PAG in the generation of headache (Welch *et al.*, 2002).

1.5.2.6 Nucleus raphe magnus

The raphe nuclei are located along the midline in the lower pons and upper medulla. Of these nuclei, the nucleus raphe magnus is implicated in both the control of VBNC neurons and the processing of trigeminal sensory information to the cortex. It receives its main input from sensory spinothalamic tract neurons via the adjacent nucleus reicularis paragigantocellularis, however a sparse direct projection from the VBNC has also been identified. Neurons in the nucleus raphe magnus and adjacent structures of the rostral ventromedial medulla (RVM) are involved in the control of nociceptive transmission. In the RVM the so-called on-cells are excited, and the so-called off-cells are inhibited, by noxious stimuli applied almost anywhere on the body surface, thus showing that they receive information from spinal and trigeminal nociceptive neurons (Hernandez & Vanegas, 2001). Indeed, the RVM has been shown to possess on- and off-cells can encode the stimulus intensity in terms of response latency and discharge rate and closely reflects spinal neuronal encoding suggesting a role in the modulation of pain information by RVM neurons.

Single unit electrophysiological recordings of cells in the raphe nuclei have indicated the presence of "pacemaker cells" which exhibit tonic discharge (Mosko & Jacobs, 1976). When this is considered with the reported functional organisation of raphe nuclei cells according to the afferent input they receive (Kirifides *et al.,* 2001), it provides weight to the theory of Eggers, that abnormally functioning cells in the raphe nuclei might underlie an excitotoxic cortical response linked to the initiation of migraine (Eggers, 2001).

The nucleus raphe magnus is an essential part of the descending analgesia system and a specific role in migraine is implicated by the discovery that naratriptan, an effective anti-migraine drug, is able to decrease activity in on-cells and increase activity in off cells in the nucleus raphe magnus (Ellrich *et al.*, 2001a). However, an indirect action of naratriptan on structures such as the PAG, which influence the activity of the nucleus raphe magnus, rather than a direct action on the nucleus raphe magnus cannot be ruled out. However, retrograde tracing studies using horseradish peroxidase have demonstrated the existence of descending pathways from the nucleus raphe magnus and nucleus paragigantocellularis to both the Vc and Vo. Furthermore, antidromic stimulation in the VBNC has been shown to activate cells in the nucleus raphe magnus (Lovick & Wolstencroft, 1983).

1.5.2.7 Gigantocellular neurons of the reticular area

The gigantocellularis neurons (or Betz cells) are located in the reticular area of the pons and mesencephalon and use acetylcholine as their neurotransmitter. The reticular formation is representative of an old neural system where neurons lie among ascending and descending tracts of all types with the ability to exert a wide range of effects on both the spinal cord and brain. There are 6 main nuclei of the reticular formation, namely the nucleus reticularis gigantocellularis, the nucleus reticularis magnocellularis, the nucleus reticularis parvocellularis, the nucleus reticularis paragigantocellularis lateralis, the nucleus reticularis paragigantocellularis and the infratrigeminal nucleus, although the term nuclei is used loosely as all have overlapping distributions. The connections of the VBNC and the brain with these nuclei have been previously reviewed (Abols & Basbaum, 1981; Newman, 1985; Van

Bockstaele, 1989). Because of their wide distribution, it is likely that these nuclei play many roles in the processing of sensory and motor function. Of particular importance in trigeminal sensitisation studies is the integration of nociceptive trigeminal input and cardiovascular responses by a regulation centre located in the most caudal portion of the reticular formation.

1.5.2.8 Locus ceruleus

The locus ceruleus (LC) is the most prominent cluster of noradrenergic neurons in the brain and is located in the grey matter of the pons. Collaterals of these neurons give rise to millions of noradrenergic terminals throughout the cortex as well as other brain regions. The nerve terminals of this system, instead of forming discrete terminals are located away from their target cells, hence central noradrenergic effects tend to be diffuse. A malfunction in the activity of the LC has been suggested to underlie both the extracranial vascular changes occurring during migraine and the process of cortical spreading depression (Lance *et al.*, 1983). More recently, a parasympathetic role in migraine has been suggested to underlie exaggerated facial flushing, lacrimation and rhinorrhea, which is supported by the increased levels of cranial venous vasoactive intestinal peptide in migraineurs during attacks. As a result of these studies, a within-brainstem interaction between the two locus coeruleus nuclei which are involved in control of pain and cranial parasympathetic outflow has been suggested to be involved in the initiation of migraine (Avnon *et al.*, 2003).

1.5.2.9 Parabrachial area

The parabrachial area, including the Kolliker-Fuse nucleus and the nucleus cuneiformis have also been implicated in the modulation of trigeminal nociception (Hayashi, 1992). Studies examining *c-Fos* expression following intra-plantar carraggeenan at both the spinal and PB levels and inflammatory edema have provided evidence for the involvement of spinoparabrachial pathway in inflammatory nociceptive processes (Buritova *et al.,* 1998). This is supported by other studies that show one of the major supraspinal targets for lamina I projection neurons is lateral parabrachial area (Spike *et al.,* 2003). Activation of the parabrachial area has also been shown to inhibit Vc neuronal responses evoked by both cutaneous and deep tissue stimulation (Chiang *et al.*, 1995).

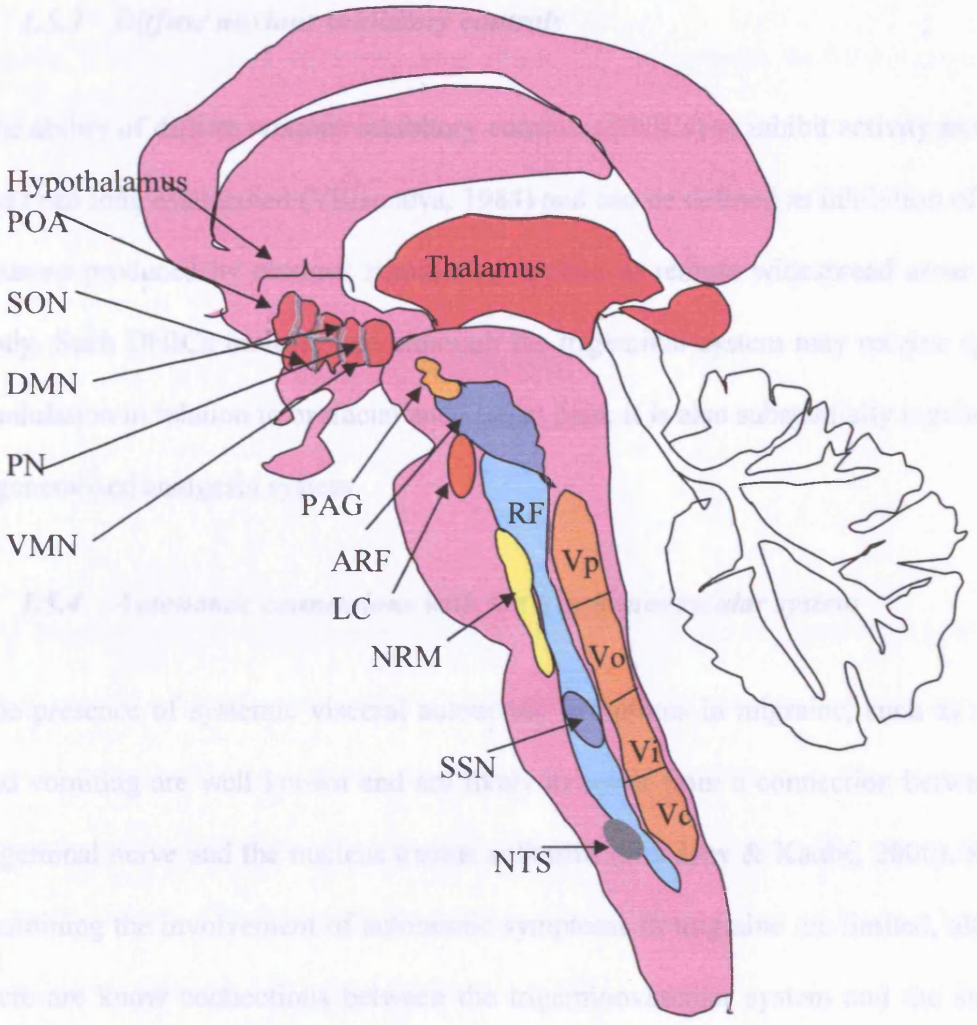


Figure 7. Brainstem structures with trigeminal brainstem nuclear complex connections

Ascending projections from the trigeminal nuclear brainstem complex reportedly have many targets
POA, Pre-optic area: SON, supra-optic nucleus: DMN, dorsomedial nucleus: PN, posterior nucleus: VMN, ventromedial nucleus: PAG, periaqueductal grey: ARF: anterior reticular formation: LC, locus ceruleus: NRM, nucleus raphe magnus: SSN, superior salivitory nucleus: NTS, nucleus tractus solitarius: RF, reticular formation.

1.5.3 Diffuse noxious inhibitory controls

The ability of diffuse noxious inhibitory controls (DNICs) to inhibit activity in the Vc has been long established (Villanueva, 1984) and can be defined as inhibition of WDR neurons produced by noxious stimulation applied to remote widespread areas of the body. Such DNICs indicate that although the trigeminal system may receive specific modulation in relation to orofacial and cranial pain, it is also substantially regulated by a generalised analgesia system.

1.5.4 Autonomic connections with the trigeminovascular system

The presence of systemic visceral autonomic symptoms in migraine, such as nausea and vomiting are well known and are likely to result from a connection between the trigeminal nerve and the nucleus tractus solitarius (Goadsby & Kaube, 2000). Studies examining the involvement of autonomic symptoms in migraine are limited, although there are know connections between the trigeminovascular system and the superior salivatory nucleus, the sphenopalatine ganglion and the nucleus tractus solitarius.

The superior salivatory nucleus receives an ipsilateral afferent input from Vc, Vo and Vp (Spencer *et al.*, 1990) as well as other brainstem structures. The pre-ganglionic parasympathetic cells in the superior salivatory nucleus then send axons to join with fibres from the superior cervical ganglion to the sphenopalatine ganglion. The sphenopalatine ganglion provides a source of vasoactive intestinal peptide and cholinergic fibres to the anterior cerebral vessels (Uddman *et al.*, 1980). However, the physiological function of the cerebrovascular vasoactive intestinal peptide innervation is currently unknown.

c-fos expression in the caudal nucleus tractus solitarius and its caudal extension in lamina X of the spinal cord was seen bilaterally in response to MMA stimulation (Hoskin *et al.*, 1999). Autoradiography studies have also confirmed that intravenously injected zolmitriptan^{3H} labels a discrete population of cells in the nucleus tractus solitarius (Martin, 1997) suggesting a possible role of this structure in the reactive response to trigeminovascular activation.

1.6 Visceral pain

The sensory pain systems described above are likely to be of central importance in many aspects of head pain. However, it is also worth considering that the dura mater may be considered as a visceral structure and as such, attention to the processing in visceral pain pathways is required for a full understanding of migraine pathophysiology. The following summary is adapted from recent reviews; (Cervero & Laird, 1999; Gebhart, 1995; McMahon *et al.*, 1995).

Characteristically, sensation of visceral pain is diffuse and poorly localised, pain is also commonly referred to skin and other areas and accompanied by autonomic and motor disturbances. While the neural mechanisms underlying visceral pain are relatively unexplored, current research suggests there are essentially two classes of visceral nociceptor responsible for the transmission of pain signals to the central nervous system. The first can be considered to be NS, responding to high threshold stimuli in the noxious range only and the second, an intensity coding type with a low threshold to natural stimuli. A further group of "silent receptors" that only respond to

stimuli in the presence of inflammation have also been proposed. The role of these "silent receptors" remains speculative, however, their function under sensitised conditions may purely aid the adaptation to normal processing.

1.7 Migraine Models

Experimental models of disease are invaluable in furthering scientific understanding and detecting pharmacological properties of new compounds that will determine their usefulness as a treatment. The basis of model development depends on clinical observations of the disease, which in primary headache syndromes such as migraine can be problematic due to the difficulty in getting patients to present at the onset of spontaneous attacks. Hence, it is useful to be able to induce attacks of migraine in patients that already suffer from the disorder, an example of which was the use of red wine. The most widely used and best characterised is the glyceryl trinitrate (GTN) model (Iversen *et al.,* 1989). The GTN model has provided considerable information on the changes in regional cerebral blood flow, cranial arterial dilatation and CGRP efflux occurring during a migraine attack. Human experimental data has made it possible to develop a number of animal models of components of migraine. No animal models are currently available that reflect the clinical picture of a full migraine attack and it is unlikely that it will ever be possible to develop such a model due to the varying pharmacology between species. However, pre-clinical research into different phases of the attack, such as aura and pain, are continually increasing knowledge of pathological events that may underlie each stage.

1.7.1 Neuronal sensitisation as a model of migraine pain

The development of models which reflect the pain phase of migraine have focused on the sensitivity seen clinically, where patients frequently report pain to normally innocuous stimuli such as head movement and light touch of areas innervated by the trigeminal nerve including large areas of the face on the side of the headache. This increased sensation is known as allodynia and is often accompanied by hyperalgesia, defined as an amplified response to noxious stimulation. Both phenomena are present in most forms of clinical pain, hence there has been a huge research effort to more fully characterise their underlying cellular mechanisms in the spinal cord, although similar investigations in the VBNC have only been evident in the last few years.

At present, it is thought the processes of peripheral and central sensitization account in some part for clinical pain sensitivity. Peripheral sensitization describes how peripheral sensory receptors may respond following exposure to algesic mediators such as might be released during times of inflammation or tissue injury. Central sensitization describes an alteration of the behavior of second order neurons within the central nervous system. The concept that CNS changes occur following noxious stimulation has now been unequivocally shown. It has been demonstrated that noxious peripheral stimuli produce changes in the sensitivity of dorsal horn neurons to further stimulation (Kenshalo *et al.*, 1982) and that a primary afferent input triggers sustained increases in central excitability (Woolf and Wall, 1983, 1986). Noxious stimulation or injury has also been shown to produce dramatic alterations in spinal cord function, including sensitization, wind-up, or the expansion of the receptive fields of spinal neurons. In addition to a neuronal hyperactivity, a variety of cellular and molecular

changes that affect membrane excitability and induce new gene expression have been proposed to occur (for review see Coderre *et al,* 1993, 1997).

A common model to study sensitization at the single cell level is the "windup" phenomenon (Woolf, 1983; Woolf & Wall, 1986; Thompson *et al.*, 1990). Action potential windup (or windup) is an increase in the number of spikes generated by a neurone after each successive stimulus (of appropriate strength) during a pulse train following activation of dorsal root thin fibres (Mendell & Wall, 1965, McMahon *et al.,* 1993; Urban *et al.*, 1994). Although sensitization can be induced by stimuli other than the one used in windup, the sensitivity to receptor blockers and the time course remain very similar, thus suggesting common underlying mechanisms.

The investigation of receptor mechanisms involved in sensitization and wind-up initially focused on the NMDA glutamate receptors (Wigstrom & Gustafsson, 1986; Faden *et al.*, 1988; Davies & Lodge, 1987; Dickenson & Sullivan, 1990; Thompson *et al.*, 1990). The voltage-dependence of NMDA receptors (where by membrane depolarization removes their Mg^{2+}-dependent block; Mayer *et al.*, 1984; Nowak *et al.*, 1984) places NMDA receptors in an ideal position to generate sensitization since the cumulative depolarization would trigger a self-reinforcing excitatory phenomenon. However, windup in dorsal horn superficial neurons has been suggested to be mediated by an NMDA receptor independent mechanism (Dickenson & Sullivan, 1999) suggesting that the role of NMDA receptors in windup might depend on the particular cell type examined or its functional conditions (Dickenson & Sullivan, 1990).

Substance P, has also been shown to have a role in wind-up and sensitization as tachykinin antagonist SR 140333 (selective for NK_1 receptors) produced a significant reduction in the rate of rise of cumulative depolarization and abolished action potential windup (Baranauskas *et al.*, 1995, 1996). However, the exact role of substance P remains is unknown, a major function of substance P in sensitization (Wiesenfeld-Hallin *et al.*, 1991) and a requirement for NMDA and NK receptor co-activation (Xu *et al.*, 1992; Thompson *et al.*, 1993) have both been suggested.

Other receptor systems suggested to have a role in wind-up and sensitization include serotonin $5\text{-}HT_{1A}$ receptors (Gjerstad *et al.*, 1996), adrenoceptors (Reeve & Dickenson, 1995), metabotropic glutamate receptors (Boxall *et al.*, 1996), opioid receptors (*Stanfa et al.*, 1992; Chapman *et al.*, 1994), TRH receptors (Chizh & Headley, 1996) and muscarinic cholinoceptors (Bleazard & Morris, 1993). Excitatory responses induced by dorsal root stimulation in the spinal cord are also under inhibitory control by both GABA and glycine receptors (Sivilotti & Woolf, 1994; Wilcox *et al.*, 1996) although it is unclear how much these inhibitory mechanisms contribute to windup.

A further possible origin of central sensitisation is an imbalance in the descending influences from the brainstem, either an increased facilitatory pathway or a decreased inhibitory tone following a pathological event such as might occur to initiate a migraine attack. Alternatively, the degree of descending inhibitory tone might be genetically set to a lower level in migraineurs, explaining why some environmental

factors, certain food types and emotional stress can trigger attacks in susceptible individuals.

The theory of peripheral sensitisation within the dura mater as a generator of central sensitisation in migraine pathology has also been investigated (for review see Burstein, 2001). One hypothesis suggests that following a cortical disturbance, as some believe to be the "trigger" phase of migraine, potassium and hydrogen ions are released into the extracellular space (Bolay *et al.,* 2002). These ions may then induce activation of peripheral nociceptors with subsequent release of mediators such as CGRP, prostanoids, bradykinin, serotonin and histamine, which further sensitise the peripheral receptors, resulting in a lowered threshold of activation. In regions where these receptors are associated with blood vessels, the pulsatile flow of blood under these sensitised conditions may then be sufficient to activate these receptors, providing one explanation for the throbbing nature of migraine pain.

Peripheral dural sensitisation has been explored in the anaesthetised rat, where an inflammatory cocktail was applied to the dura mater and the response of second-order neurons in laminae IV and V of the Vc to dural and facial stimulation examined. Profound sensitisation of both the dural and FRF was seen to thermal, mechanical and electrical sensitisation that lasted for up to 10 hours (Burstein *et al.*, 1998). This corresponded well with data from a later clinical study that assessed pain thresholds during a migraine attack with the finding that more than 70% of patients developed cutaneous allodynia ipsilateral to the headache (Burstein *et al.*, 2000). The study also found that at later time points, allodynia could also be detected in the contralateral head and forearms, which led the authors to suggest that not only did peripheral

sensitisation in the dura result in central sensitisation of Vc neurons but also of brainstem neurons such as third order neurons in the thalamus. Another significant finding of the study was the time-dependence (i.e. the duration that individuals had suffered from migraine) in the development of cutaneous allodynia implicating that the strength of the synapses that facilitate central sensitisation increases after repetitive use.

1.7.2 Electrophysiology

1.7.2.1 Human studies

Electrophysiology has been used in humans to determine the role of the VBNC in trigeminal function during a migraine attack by the use of a technique known as the blink reflex. In this reflex, electrical pulses or radiant heat is used to stimulate the supraorbital nerve, which activates primary afferents, synapsing in the Vc to exert the efferent blink effect via the facial nerve. The resulting electromyogram response determined by non-invasive surface electrodes, has three components named R1, R2 and R3. Both R1 and R3 can be triggered by stimuli in the innocuous range (Ellrich *et al.*, 2001b), where as R2 can by elicited by selective activation of nociceptors by the use of a newly developed concentric stimulating electrode which only stimulates the superficial A-δ and C-fibres (Kaube *et al.*, 2000). Several studies have now demonstrated a facilitation of this nociceptive-specific component of the blink reflex during migraine attacks, indicating central sensitisation of nociceptive circuitry during migraine (Katsarava *et al.*, 2002; de Tommaso *et al.*, 2002).

1.7.2.2 Animal studies

Electrophysiological recordings in anaesthetised animals are an extremely useful tool for assessing how either a single neurone or a population of neurones may respond to a specific stimulus. With relevance to migraine pathophysiology, this has been carried out in both trigeminal ganglion and VBNC neurons and more recently on brainstem neurons. Further details of methodology are provided in Chapter 2.

1.7.3 Neuropeptide release

In human studies, CGRP levels are elevated in the jugular vein during a migraine attack (Goadsby, 1990). This corresponds well with animal data, where stimulation of the superior sagittal sinus results in the release of CGRP in both cat and rat (Zagami *et al.*, 1990). Stimulation of the trigeminal ganglion increases both CGRP and SP levels in humans and cats (Goadsby *et al.*, 1988), which given the failure of SP antagonists in migraine, may indicate better predictability in pre-clinical models could be obtained by specific stimulation of the durovascular afferents rather than the trigeminal ganglion.

Recently, an *in vitro* method of examining the release of neuropeptides and other mediators in the dura mater has been described (Ebersberger *et al.*, 1999). In these studies, a rat skull was divided in two and bathed in a synthetic interstitial fluid allowing the effects of trigeminal ganglion stimulation to be assessed by use of enzyme immunoassays on the superfusate collected. It is also possible in this model to introduce drugs and chemical mediators into the synthetic interstitial fluid and assess effects.

1.7.4 Plasma protein extravasation (PPE) and neurogenic inflammation

The possible involvement of a neurogenic inflammation within the dura mater during the pain phase of migraine was first suggested by Moskowitz (1984). This group showed that following electrical stimulation of the trigeminal ganglion, leakage of plasma proteins could be detected in the rat dura and retina, as measured by iodine-radiolabelled albumin. However, there is currently no evidence of plasma protein extravasation occurring clinically during acute attacks of migraine or cluster headache when MRI scanning of patients (Nissila *et al.*, 1996) or retinal angiography (May *et al.*, 1998) has been carried out. Stimulation of the trigeminal ganglion, also results in a neurogenic vasodilatation, with resultant increases in cerebral and extracerebral blood flow observed in several species, including human (Goadsby, 1997). In rats and cats, this effect can be blocked by the CGRP antagonist, $CGRP_{8-37}$, indicating it is the result of CGRP release from trigeminal sensory nerves (O'Shaughnessy & Connor, 1994; Wei *et al.*, 1992).

Interestingly in rat models of neurogenic inflammation induced by trigeminal ganglion stimulation, neurogenic vasodilatation and plasma protein extravasation have been shown to be differentially affected by 5-$HT_{1B/1D}$ agonists (Shepheard *et al.*, 1997). It appeared that inhibition of plasma protein extravasation was due to an action through the 5-HT_{1D} receptor where as inhibition of dural vasodilatation was due to an action at the 5-HT_{1B} receptor. Furthermore, the stimulation parameters required to elicit a neurogenic vasodilatation were much lower than stimulation parameters required to elicit plasma protein extravasation indicating that activation of different trigeminal

obtained by using laser Doppler studies. Details of this technique are discussed further in Chapter 2.

1.7.6 Molecular markers

The investigation of molecular events underlying the neuroplastic response to nerve stimulation and injury has provided significant advances in the understanding of various pain states in recent years. In particular, expression of the *c-fos* gene following noxious but not innocuous stimulation has been observed in the spinal cord and is considered a useful marker of neuron activation (Hunt *et al.*, 1987; Munglani & Hunt, 1995; Naranjo *et al.*, 1991; Sagar *et al.*, 1988). Fos is an acronym of the words: Finkel, Osteogenic and Sarcoma, used to describe the gene encoded by the Finkel-Bislis-Jinkins murine osteogenic sarcoma retrovirus. This "viral-*fos*" and other immediate early genes were shown to be capable of inducing cellular proliferation and as such, are now known as proto-oncogenes. Many of these proto-oncogenes have now been identified in cells and share the property of rapid induction following stimulation without the need for protein synthesis (Evans, 1995). They are thought to regulate long-term cellular function by altering the downstream expression of target genes, through interaction with several second messenger systems, following an increased intracellular calcium concentration induced by the action of various neurotransmitters (Armstrong & Montminy, 1993; Lerea & McNamara, 1993; Peunova & Enikolopov, 1993).

The induction of cellular *fos* (*c-fos*) has proved to be an extremely useful marker of

those cells in the spinal cord that are activated by peripheral noxious stimulation (Sagar *et al.,* 1988). This is supported by the observation that *fos*-expression is restricted to laminae, I, II and V of the spinal cord, which receives input from Aδ and C-fibres associated with the transmission of noxious signalling. *c-fos* mRNA can be observed minutes after a stimulus and the c-*fos* protein, 1-2 hours after stimulation, allowing large sections of brain and spinal cord to be analysed for either *fos* mRNA or protein and correlated with anatomical pathways known to be activated by a particular stimulus.

The long-term cellular effects of *c-fos* activation are thought to be mediated through the formation of a complex between c-*fos* protein and c-*jun* protein (a product of another immediate early gene). This protein complex, in turn, forms part of the AP1 transcription factor complex, which is able to alter the expression of various transcription genes, ultimately resulting in a modification of cellular behaviour (Chiu *et al.*, 1988; Halzonetis *et al.*, 1988). Hence, in addition to providing a marker of neuronal activation, c-*fos*-expression is likely to provide an indication of the location of a neuroplastic response to noxious stimulation.

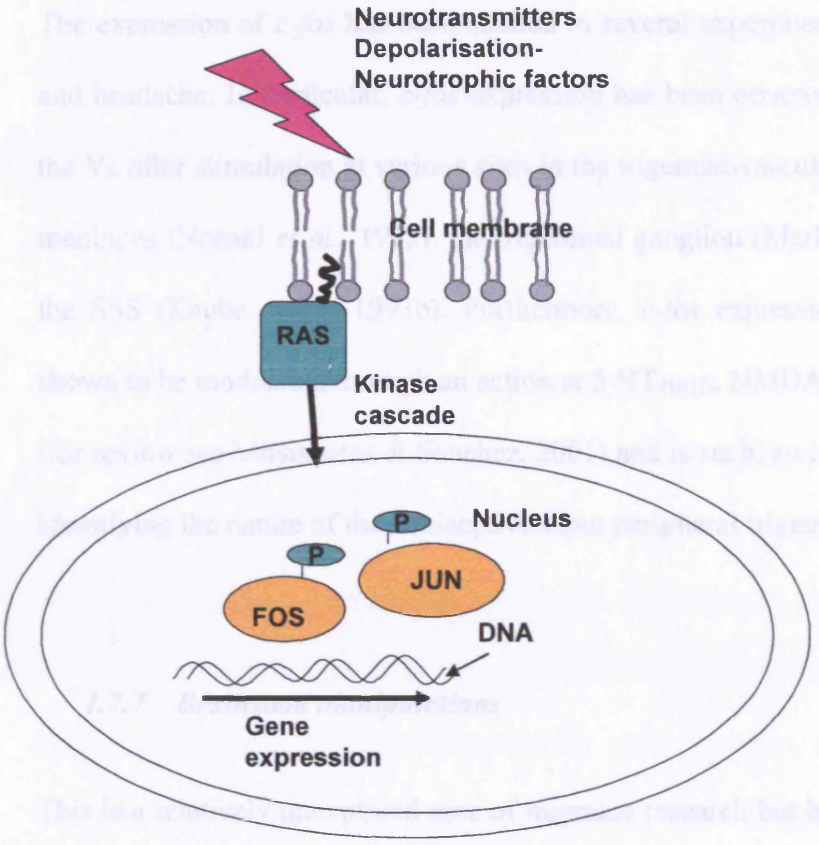


Figure 8 *c-Fos* expression

Factors such as neurotransmitters and hormones lead to an influx of intracellular calcium which activates kinase cascades which ultimately result in the phosphorylation of transcription factors such as fos and jun. Fos is then able to form a heterodimeric complex with other nuclear proteins of the Jun family. This complex then binds to DNA, where it exerts its effects on expression of target genes, resulting in long-term cellular changes. The action of the fos-jun complex is then eventually terminated by the action of phosphoprotein phosphatases.

The expression of *c-fos* has been studied in several experimental models of migraine and headache. In particular, *c-fos* expression has been observed in lamina 1 and 2 of the Vc after stimulation at various sites in the trigeminovascular system, including the meninges (Nozaki *et al.*, 1992), the trigeminal ganglion (Markowitz *et al.*, 1988) and the SSS (Kaube *et al.*, 1993b). Furthermore, *c-fos* expression in the Vc has been shown to be modulated through an action at 5-$HT_{1B/1D}$, NMDA and glutamate receptor (for review see Mitsikostas & Sanchez, 2001) and is such, an extremely useful tool for identifying the nature of the nociceptive input peripheral trigeminal structures.

1.7.7 Brainstem manipulations

This is a relatively unexplored area of migraine research but has huge potential in the development of models with relevance to the pain phase, the trigger phase and premonitory phase of migraine. Recent exciting new data showed the activity of Vc neurons as assessed by electrophysiology could be modified by selective blockade of P/Q type calcium channels in the PAG using a micro-injection system (Knight *et al.*, 2002). Further exploration of this area is possible using the technique of ionophoresis, where an electrode containing micropipettes of drug solutions can be inserted into the area of interest and the required drug solution expelled (Storer & Goadsby, 1997).

1.7.8 Models of aura- cortical spreading depression (CSD)

The phenomenon of cortical spreading depression (CSD) has developed from the observations of Lashley (1941) who estimated that his own visual symptoms

preceding migraine were spreading over the cortex at rate of about 3mm each minute. These observation correlated well to the description by Leao (1944) of a progressive shutdown of cortical function with inhibitory waves moving slowly over the cortex at a speed of 2-3mm each minute and suppressing normal neuronal activity in the animal brain (Leao, 1944).

Subsequent blood flow studies in migraine patients have shown that a "wave" of oligaemia spreads over the cortex at a similar speed (Lauritzen, 1983), which led to the conclusion that spreading oligaemia was responsible for the spread of fortification spectra and other neurological symptoms described as aura (Lauritzen, 1994). Extensive animal studies have since ensued, with reports of models of progressive inhibitory neuronal waves passing over the cerebral cortex, preceded by a transient excitation, following a stimulus such as application of potassium chloride to the cortex. However, correlation of CSD in animals with aura phenomena observed in patients is only speculative. The importance of studying aura, given that only a small number of patients report aura like symptoms is uncertain. However, it is possible that the manifestation of visual symptoms is related to a threshold and these cortical waves are the trigger for both migraine with aura and migraine without aura but do not reach a sufficient level to trigger visual symptoms in migraine without aura patients.

1.7.9 Functional imaging

The technique of positron emission tomography (PET) provided evidence for the involvement of the rostral brainstem during spontaneous migraine attacks in migraine without aura sufferers (Wellier *et al.*, 1995). The study also showed that the activation

persisted after treatment aborting the head pain, indicating a fundamental role of the area in migraine pathophysiology, rather than simply a response to pain. Further functional imaging studies would be extremely useful in migraine research, however the expense and limitation of equipment severely limits the technique.

1.8 Drug treatments for migraine

An accurate diagnosis is crucial in the treatment of migraine, however given the large array of headache disorders and the varied symptoms associated with each, migraine is estimated to highly under-diagnosed. When a patient presents with migraine symptoms, other co-morbid illness should be investigated to ensure drug interactions are not underlying the onset of headache symptoms. Factors such as pregnancy, menopause and the use of oral contraceptives, also have significant implications for migraine and care needs to be taken to consider this. Once a diagnosis of migraine has been established, patients need to be educated about their condition and the avoidance of triggers, in order to maximise the efficacy of pharmacotherapy. In general, migraine pharmacotherapy involves either or both prophylactic and acute medication depending on the frequency and severity of attacks.

Migraine triggers	
Diet	Alcohol
Sleep	Alteration in pattern
hormonal changes	Menstruation, Oral contraceptives
environment	Climate changes
stress	Anxiety

Table 4 Migraine triggers

1.8.1 Pre-emptive treatment

In those patients who can predict the onset of a migraine attack by the presence of premonitory symptoms, administration of 30-40mg domperidone has been found to be effective in preventing development of the headache in some patients (Waelkens, 1981).

1.8.2 Anti-Emetics

Many migraine patients suffer nausea and vomiting, which they may find more distressing than the associated head pain; hence, anti-emetics should be a consideration in the drug treatment for migraine. Both the chemoreceptor trigger zone (CTZ), located in the fourth ventricle and the vomiting centre, which receives input from the CTZ, as well as other higher centres and the upper gastrointestinal tract are the site of action for anti-emetics. The CTZ lies outside the brain-barrier and hence is subject to activation by many toxins. It is the site of action of anti-emetics such as domperidone, metoclopramide and the phenothiazines, which all have dopamine antagonist properties. The vomiting centre is also rich in muscarinic, cholinergic and histamine receptors that are also the site of action of anti-emetics, such as

butyrophenones and phenothiazines. There is limited clinical data concerning the efficacy of anti-emetics in the treatment of migraine but as gastric stasis and delayed gastric emptying are commonly associated with migraine (Boyle *et al.*, 1990), pro-kinetic anti-emetics, which promote gastric emptying, are the first choice treatment.

1.8.3 Acute treatment

1.8.3.1 Ergot Alkaloids

For over a century, ergot alkaloids were the drug of choice for the treatment of head pain with medical reports of their efficacy as early as 1883 (Eulenburg, 1883). Ergots are powerful vasoconstrictors; however, their effects are long lasting and not specific to cranial vessels. Their wide action on adrenergic and dopaminergic systems results in a side effect profile of dysphoria, nausea, emesis and cardiovascular effects, which limits their therapeutic use. However, they are still used sparingly in migraine, in certain specific settings (Tfelt-Hansen *et al.*, 2000).

It is largely due to the potent vasoconstrictor action of the ergot derivatives that the vascular theory of migraine was formulated by Graham & Wolff in 1938. Further study of ergot derivatives identified other mechanisms of actions, such as the inhibition of neurogenic inflammation (Saito *et al.*, 1991) and central inhibition of trigeminal pain pathways (Goadsby, 1991; Hoskin *et al.*, 1996), all of which has significantly advanced our understanding of migraine pathophysiology. However it was the studies of Saxena (1972), using methysergide, an ergot derivative and 5-HT antagonist that generated a significant advance during the last decade in acute migraine therapy, by providing the rationale for the development of the triptans.

1.8.3.2 Triptans

The late eighties saw the development and marketing of the first 5-HT $_{1B/1D}$ agonist, sumatriptan for the specific treatment of migraine (Humphrey *et al.*, 1990). Sumatriptan was not only a breakthrough treatment but it also altered clinical and scientific opinion on the characterisation of migraine as a recognised disease. However, there were significant problems with oral bioavailability, slow onset of action, brain penetration and headache recurrence. The availability of a subcutaneous injectable form of the drug, and later as a suppository and intranasal spray, removed many of these problems. However, a significant number of patients still do not respond to sumatriptan and many are left with some residual head pain. A further problem with sumatriptan is the constriction of peripheral vessels and the contra-indication in-patients with history of ischemic heart disease. As a result many so-called "second-generation triptans" have been developed, notably naratriptan, rizatriptan, zolmatriptan, eletriptan, almotriptan and frovatriptan each with a literature suggesting benefits in onset of action, recurrence rate or adverse event profile, placing the responsibility on the physician to determine the right triptan for the patient.

Much work has been carried out to determine the mechanism of the anti-migraine activity of the triptans. However, until recently, work has been hampered by the lack of highly selective 5-HT$_1$ agonists. The development of highly specific polyclonal antibodies to both the 5-HT$_{1B}$ and the 5-HT$_{1D}$ receptors has allowed determination of their anatomical distribution. In the human, a rich density of 5-HT$_{1B}$ but not 5-HT$_{1D}$ receptors has been identified in the medial layer of the MMA (Nilsson *et al.*, 1999).

Both 5-HT_{1B} and 5-HT_{1D} receptors have been identified on human trigeminal ganglion, although in studies on trigeminal nerve fibres, only 5-HT_{1D} immunoreactivity was observed, with immunoreactivity observed on both peripheral projections to the dura mater and central projections to the Vc (Longmore *et al.*, 1997). However, in the rat, autoradiographic receptor mapping studies have identified 5-HT_{1B} receptors in the spinal trigeminal tract (Bruinvels *et al.*, 1994) and 5-$HT_{1B/1D}$ receptors on cell bodies of trigeminal neurons, Vc and in the spinal cord at the level of C_1 / C_2.

Several possible sites of action for the triptans have been identified and an effect at all sites is likely to contribute to the overall anti-migraine activity. In the first instance, a selective cranial vasoconstrictor effect has been demonstrated for all available triptans, lending weight to the theory developed by Wolff in the 1940's that during an attack, cranial vessels become excessively distended resulting in activation of the trigeminal sensory fibres innervating them. Of the triptans, sumatriptan has been studied most extensively for it's vasoconstrictor effect, which is now generally accepted to be mediated through the 5-HT_{1B} receptor, and hence it is likely that the observed craniovascular selectivity seen over coronary arteries may be explained by the relative density of 5-HT_{1B} on cranial and coronary vessels (Villalon *et al.*, 1999).

Activation of the trigeminal nerve has been extensively studied in animal models as detailed earlier, with the aim of further understanding how this system may dysfunction in migraineurs. The triptans have been tested for efficacy in peripheral models such as PPE, intravital microscopy and neurogenic inflammation and in all cases have inhibited trigeminal nerve activity. However, it should be remembered that

other classes of drugs such as the neurokinin-receptor antagonists, are also able to inhibit PPE but are not effective in the treatment of migraine. Naratriptan and Zolmitriptan are also able to block electrophysiological recordings and *c-fos* expression in the Vc following stimulation of the dural receptive field (Goadsby & Hoskin, 1996; Goadsby & Knight, 1997). This effect appears specific to the trigeminal system as no effect on lumbar dorsal horn responses linked to sciatic nerve stimulation is seen following triptan administration (Cumberbatch *et al.*, 1997).

Further to an action through the 5-$HT_{1B/1D}$ receptors, many of the triptans also have affinity for the 5-HT_{1F} receptor. The selective 5-HT_{1F} receptor agonist, LY334370 (Eli Lilly) has recently been developed and has been found to be effective at inhibiting both neurogenic vasodilatation and firing of second order neurons within the Vc, without any vasoconstrictor effects on cerebral arteries in vitro (Shepheard *et al.*, 1999). It has also proved successful in the treatment of acute migraine in a randomised controlled trial (Goldstein *et al.*, 2001).

1.8.3.3 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory compounds have been used since the time of the Hippocrates, 2000 years ago for the treatment of pain (Butler, 1998; Levesque & Lafont, 2000). Since the isolation of acetylsalicylic acid from extracts of willow bark, many other non-steroidal anti-inflammatory drugs' (NSAID's) have been synthesised with different properties. However, all NSAIDs are weak organic acids, with the main mechanism of action being prevention of the prostaglandin synthesis from arachidonic acid with anti-inflammatory, analgesic and anti-pyretic activity. The mechanism of

action of NSAIDs in headache is not fully understood but in animal models, they are effective they have been reported to inhibit both neurogenic inflammation and firing of Vc neurons, suggesting a combination of central and peripheral mechanisms (Buzzi *et al.*, 1989; Kaube *et al.*, 1993a).

Initial trials using NSAID's for the treatment of migraine were limited in success, however this was later discovered to be due to the lack of absorption due to the delayed gastric emptying seen during migraine. Recent trials where NSAIDs have been co-administered with anti-emetics, have shown the combination to be as effective as the triptans (Diener, 1999), warranting further investigation to the mechanism of action of NSAIDs and the role of prostanoids in migraine. There has also been recent interest in the new class of NSAIDs, which selectively inhibits the COX-2 enzyme and are associated with a lower risk of gastrointestinal side-effects. Preliminary trials for migraine with rofecoxib (a COX-2 inhibitor) have indicated that while it is effective in long-lasting reduction of headache and in reducing photophobia, phonophobia and nausea (Silberstein *et al.*, 2004), it offers no superiority over ibuprofen (Misra *et al.*, 2004). However, a combination of rizatriptan and rofecoxib reduced headache recurrence rates, was well tolerated and was suggested to be more effective than the use of rizatriptan alone (Krymchantowski & Barbosa, 2002).

1.8.3.4 Opioids

Opioids describe a group compounds with morphine like activity. Analgesia induced by this class of compounds occurs at several spinal and supraspinal sites. Agonists acting at post-synaptic mu receptor sub-type can selectively inhibit nociceptive

transmission and agonists acting at pre-synaptic kappa, delta as well as the mu receptors inhibit the release of transmitters involved in pain transmission (such as glutamate and substance P). Opioids are also able to cause direct hyperpolarization of post-synaptic cells in the trigeminal nucleus caudalis and dorsal horn, hence reducing pain transmission into the brainstem (Storer *et al.,* 2003; Williamson *et al.,* 2001). The use of opioids to treat migraine is controversial and due to their addictive properties, it is recommended that use by restricted to patients not responding to other medication, pregnant patients or patients who are unable to take headache medication due to drug interaction with other treatment regimens (Silberstein & McCrory, 2000).

1.8.3.5 Miscellaneous drugs

A wide range of drugs other than triptans, ergots or NSAIDs are currently used for the acute treatment of migraine however, there is not significant evidence from controlled trials to indicate superiority over placebo for many of them. Isometheptene, a sympathomimetic amine, is widely used in countries such as the United States of America for the treatment of mild migraine, largely due to its safer cardiovascular profile than the triptans. Intranasal lidocaine, corticosteroids, magnesium, calcium antagonists, nitric oxide synthase inhibitors (Blanda *et al.,* 2001; Lassen *et al.*, 1998, 2003) and beta-blockers have also been explored as acute treatments with little success to date.

Despite the lack of success in the generation of a safer, efficacious migraine treatment, clinical trials have aided in the development of understanding in migraine. The failure of the NK-1 receptor antagonists (Diener *et al.,* 1995, 2003; Connor *et al.*, 1998;

Goldstein *et al.*, 1997) and similarly the endothelin antagonists (Brandli *et al.*, 1996; Clozel *et al.*, 1994) to work in acute migraine, despite efficacy in some pre-clinical models, has necessitated the development of new animal models.

1.8.4 Prophylactics

The use of preventive treatment in migraine is usually considered for patients where attacks are occurring three times a month or more, or for patients suffering less frequent but more severe attacks, or where a specific trigger exists for attack, e.g. indomethacin is effective in preventing an attack if taken before exercise in those patients where exercise is a trigger factor. The advances in prophylactic treatment are significantly behind those made in acute treatment and no pattern has been established of how patients respond to the available medications, emphasising the likelihood that migraine is indeed a multifactoral disease. Hence, migraine prophylaxis is largely a case of trial and error.

1.8.4.1 β-blockers

Following the introduction of β-blockers for cardiac disorders in the 1960's, their effectiveness in reducing migraine frequency was serendipitously discovered shortly after (Rabkin *et al.*, 1966). They are currently the first choice for migraine prophylaxis and between 60 and 80% effective in reducing the frequency of attacks by half. The mechanism of action of β-blockers is unknown and despite distinctive pharmacological profiles of the different drugs in this class such as blood brain-barrier penetration and receptor selectivity, (at least three types of β-adrenergic receptor sub-types are known to exist), no advances have been made in this area. Discovery of the

mechanism of action is further hampered by the lack of animal models of prophylaxis.

1.8.4.2 Anti-convulsants

The use of anti-convulsant medication for migraine prophylaxis is becoming increasingly common due their proved effectiveness in clinical trials. The anti-convulsant activity of most of the drugs in this class is thought to be due to a state-dependant blockade of voltage dependant sodium or calcium channels or an ability to enhance the activity of GABA at $GABA_A$ receptors through a chloride conductance (Rogawski & Porter, 1990; MacDonald & McLean, 1986).

1.8.4.3 Serotonin antagonists

The ergot derivative, methysergide, was the first available treatment for migraine prophylaxis with reasonable efficacy in reducing migraine frequency, however its significant side effect profile restricts its use. Methysergide is a potent 5-HT_2 antagonist at the 5-HT_{2B} and 5-HT_{2C} receptor but also acts at an agonist at the 5-HT_{1B} receptor. Its mechanism of action is still debatable and theories include a vasoconstrictor activity within the carotid vascular bed (Saxena & Verdouw, 1984) and pre-synaptic inhibition of CGRP release from perivascular sensory nerves (Moskowitz, 1992).

1.8.4.4 Antidepressants

Anti-depressants of all classes (monoamine oxidase inhibitors, tricyclics and selective serotonin re-uptake inhibitors) have been assessed in both migraine and headache

prophylaxis. Due to the wide range of actions throughout the different classes of anti-depressant drugs, their mechanism of headache prophylaxis is uncertain but it does not result from treating masked depression as the response to treatment is considerably more rapid than the expected onset of any anti-depressant effect (Couch *et al.*, 1976). However most antidepressants that are clinically effective for headache prophylaxis can either inhibit 5-HT reuptake or are antagonists at the 5-HT_2 receptor (Richelson, 1990).

1.8.4.5 Botulinum toxin A

Clinical data has demonstrated that Botulinum toxin A (BTX-A) is an effective and well-tolerated therapy for the prevention of migraine and other headache disorders (Blumenfeld *et al.*, 2004) Injection of BTX-A directly affects neuromuscular signalling processes by inhibiting the vesicular release of the acetylcholine (ACh) neurotransmitter at the neuromuscular junction. Although inhibition of neuromuscular activity may alleviate a portion of the pain associated with headache disorders, it does not fully explain the pain-relief mechanisms mediated by BTX-A. It has now been suggested that BTX-A may interact with several other neuronal-signalling pathways, although the exact mechanisms remain elusive. Current data suggests that BTX-A modifies the sensory feedback loop to the central nervous system by blocking intrafusal fibres, resulting in decreased activation of muscle spindles. This effectively alters the sensory afferent system by reducing the traffic along Ia spindle afferent fibres (Rosales *et al.*, 1996). BTX-A also appears to inhibit the release of glutamate and CGRP from primary afferent nociceptive fibres, reduce the firing of wide-dynamic-range neurons within the dorsal horn of the spinal cord, and reduce the activity of central nociceptive neurons, as demonstrated by decreased expression of

immediate early genes (*c-Fos*) after nociceptor stimulation (Aoki, 2002). A reduction in afferent sensory activity coming from pericranial and cervical muscles, and inhibition of peripheral and central trigeminal sensitization, may be the potential mechanisms by which BTX-A exerts its therapeutic effect in migraine, tension-type headache, and other primary headache disorders (Burstein *et al.*, 2000).

1.8.4.6 Miscellaneous drugs

Prophylaxis has also been explored with histamine, clonidine (α-2-adrenoreceptor agonist), the herbal treatment feverfew, magnesium and amantadine (for review see Siberstein *et al.*, 2001). Although trials have been small and not statistically powered, some efficacy has been suggested with magnesium and feverfew. Interesting laboratory data has demonstrated a possible reduced brain tissue magnesium concentration during a migraine attack and a reduced magnesium content in erythrocytes, serum, CSF, and saliva both during an attack and interictally (Mauskop *et al.*, 1998; Peikert *et al.*, 1996).

1.9 Genetics of Migraine

Within the general population, 12-16% of the population suffers recurrent attacks fulfilling IHS criteria to be classified as migraineurs (for review see Ferrari & Hann, 2001). The question that genetic studies then need to address is the susceptibility of certain individuals to this trigger. The inheritance of migraine was reported as early as the seventeenth century (Willis, 1682) but despite intensive research over the last forty years, it still remains an area of controversy. The lack of clarity may be due to the

methodological difficulties in examining large patient groups and in many inheritance studies, subjects are selected from clinical populations. Family histories are also taken from the subjects without relatives being interviewed on a first hand basis, which given the subjective nature of the disease is unlikely to provide the degree of accuracy required for validity. Further complicating factors in these investigations is the high prevalence of migraine, hence a positive family history cannot necessarily be assumed to indicate a genetic component.

Despite the issues above, several genetic epidemiological studies have shown an increased familial risk amongst first-degree relatives in the development of migraine without aura of 1.9 and those with migraine with aura of 3.8 when compared to the general population (Russell & Olesen, 1995). Of greater significance in the assessment of the contribution of genetic and environmental factors to migraine, is the use of twin studies. The basis of twin studies is the comparison of monozygotic (MZ) twins, who share all of their genes and dizygotic (DZ) twins, who like ordinary siblings share 50% of their genes by descent. In a recent study Ulrich & Gervil (1999), found a higher concordance rate for MZ rather than DZ twins in both migraine with aura and migraine without aura, however as the concordance rate was less than 100%, multifactoral inheritance (i.e. environmental and genetic factors) is likely to influence the development of the disease. Other studies have also shown that genetic heterogeneity (i.e. the development of the migraine phenotype resulting from different genotypes) is likely to be responsible for both migraine without aura and migraine with aura (Ulrich *et al.*, 1997).

Due to the likely multifactoral nature of migraine without aura and migraine with aura,

little progress has been made in the identification of candidate genes. However, significant advances have been made in the study of familial hemiplegic migraine (FHM), a rare autosomal dominant subtype of migraine with aura. Patients suffering with FHM, as well as having either typical migraine without aura or migraine with aura, experience attacks characterised by migrainous head pain, nausea, photophobia or phonophobia accompanied by hemiparesis and in some patients, progressive cerebellar ataxia has also been reported (Hann *et al.*, 1997). In the early 1990's, genetic studies identified a linkage of FHM patients to chromosome 19p13 markers, in over 50 % of cases (Ophoff *et al.*, 1994). Further studies identified the FHM candidate region as a α_{1A} subunit gene (CACNA1A) for the P/Q voltage gated calcium channel, specifically transcribed in the cerebellum, cerebral cortex, thalamus and hypothalamus (Ophoff *et al.*, 1996). The potential of a dysfunctional calcium channel in these areas is of particular relevance to migraine due to the involvement of these structures in controlling descending modulation of areas receiving trigeminal afferent signals. Central 5-HT release, which has been strongly implicated in migraine pathophysiology, is also controlled by these neuronal calcium channels and hence an inappropriate 5-HT level may be involved in the triggering of an attack (Codignola *et al.*, 1993).

Despite the demonstration of mutations in chromosome 19 as part of the migraine spectrum, additional genes need to be investigated to fully explain the susceptibility of some individuals to migrainous symptoms. The last few years have seen an increasing interest in this area and several other possibilities have been investigated e.g. increased frequency of the dopamine D2 receptor allele (Peroutka *et al.*, 1997), X-chromosome linked factors (Nyholt *et al.*, 1998). There is also significant co-morbidity of migraine

with diseases such as epilepsy, Raynaud's phenomenon and several psychiatric disorders, which may provide future insights to genetic disposition factors. At present however, the identification of different mutations between families, on genes such as CACNA1A significantly hamper the development of transgenic animal models.

1.10 Summary & Aims

The literature reviewed in this chapter suggests that migraine is an extremely complex neurobiological disorder that can be influenced by many factors. Thus, the study of the disease is generally considered in different phases, as such this thesis has focused on investigation of the part of the migraine attack that results in the actual head pain and associated facial allodynia and hyperalgesia. While it is likely that central brain structures are crucially involved in this process, there is also considerable evidence to suggest that hyperexcitability of the trigeminovascular system is a consequence of migraineous pathology. This thesis has focused on possible mechanisms involved in the sensitisation of the Vc and sought to characterise whether the differential response to dural stimulation under sensitised and non-sensitised conditions is related to the clinical difference between migraineurs and non-migraineurs and details of each study are summarised in more details below.

1.10.1 Study 1 –GTN

Glyceryl trinitrate (GTN) infusion in both migraine patients and healthy volunteers induces an immediate and often pulsatile headache in nearly all subjects, which in healthy volunteers typically subsides at the end of the infusion (Iversen *et al.*, 1989). However, a sub-population of migraineurs reportedly develops a migraine headache

that resembles their own spontaneous migraine headaches, approximately 4 to 5 hours after the start of the infusion (Christiansen *et al.*, 1999). Hence, GTN may trigger a sequence of intracellular events that are responsible for the generation of some of the pathophysiological process that occur during spontaneous migraine attacks. The aim of this study was to investigate the effect of GTN on components of the trigeminovascular system using intravital microscopy, single unit electrophysiological recordings in the Vc and *c-fos* immunohistochemistry.

The underlying susceptibility migraineurs appear to have to GTN infusion compared to non-migraineurs has also been examined by looking at the effect of GTN following the induction of central sensitisation. While central sensitisation is generally accepted to play a role in the clinical features of migraine (Burstein *et al.*, 2000), the origin of sensitisation is unknown with arguments for both a peripheral (Burstein *et al.*, 2001) and central driven mechanism (Knight *et al.*, 2002). However, both arguments agree that central sensitisation at the level of the Vc is a likely consequence, as such, in these studies, inflammatory mediators were injected into the face in areas innervated by the second division of the trigeminal nerve. As described earlier, these primary afferents synapse in the Vc and show considerable convergence with primary afferent fibres innervating dural blood vessels, hence it is likely that peripheral sensitisation of primary afferent V_2 afferents will generate central sensitization in an area where afferents from the dura, synapse.

1.10.2 Study 2-Prostaglandins

PGs were implicated in the pathology of migraine as long ago as 1960's, when PGE_1 infusion in healthy volunteers was found to induce headache and facial flushing in two

out of three subjects, with one subject, who had no history of migraine, complaining of visual symptoms preceding the headache (Bergstrom *et al.*, 1965). This study was later expanded and PGE_1 infusion was reported to induce a pulsating headache in all eight subjects studied. In some subjects this was accompanied by migrainous features such as aura-like symptoms, temporary unilateral localisation of head pain, abdominal pain and nausea (Carlson *et al.*, 1968).

In naturally occurring migraine attacks, PGs have also been identified in the saliva of patients (Vardi *et al.*, 1983). Furthermore, a significant increase in the plasma concentration of PGE_2 during the headache compared to the headache free period has been reported in sufferers of menstrual migraine (Nattero *et al.*, 1989). Despite these findings, and the known effectiveness of NSAIDs, which have been reported to be almost as efficacious as sumatriptan in the treatment of acute migraine pain (Diener *et al.*, 1999), it is only in the last few years that experimental exploration examining the involvement of PGs in generating head pain has been carried out. Hence, the aim of studies in this part of the thesis was to investigate whether PGs can sensitise primary afferent fibres innervating the MMA resulting in a facilitated response of Vc neurons, leading to the generation of central sensitisation, as assessed by *in vivo* electrophysiology.

1.10.3 Study 3-Wind-up

In this study, we have thus explored whether temporal summation or "wind-up" occurs in second-order Vc neurons following activation of dural afferent using an electrical stimulation protocol. Wind-up of cells receiving C-fibre input, but not Aδ-fibre input, from cutaneous areas is a well-known electrophysiological phenomenon and can

generate an experimental state of central sensitisation (Woolf, 1996). The dura mater is almost exclusively innervated by Aδ and C-fibres (Andres *et al.,* 1987) and while cutaneous Aδ- fibres do not show wind-up, whether visceral Aδ- fibres are capable of "winding-up" is not known. Hence, both the Aδ and C-fibre component of the response were examined for wind-up and compared to the response of Vc neurons receiving cutaneous input form the face. Additionally, as previously discussed for study 1, central sensitisation has been reported to occur during migraine and generate the facial hypersensitivity reported by migraine patients during an attack (Burstein *et al.* 2000). Hence, as part of this study, we have examined whether the experimental induction of central sensitisation alters the wind-up response, as previously reported to occur in the dorsal horn (Herrero *et al.,* 1996; Stanfa *et al.*, 1992).

2 Methods

Methodological studies in this thesis largely used the technique of *in vivo* electrophysiology to investigate the activity of second order neurons within the Vc. However, the techniques of intravital microscopy and immunohistochemistry were also used to support electrophysiological data.

2.1 Electrophysiology and Intravital microscopy

2.1.1 Preparatory surgery and anaesthesia

All studies were conducted under terminal anaesthesia in accordance with a project license issued by the Home Office of the United Kingdom under the Animals (Scientific Procedures) Act, 1986.

Male Sprague-Dawley rats (280-400g) were anaesthetised using isoflurane (3-5% in oxygen; Abbott Laboratories Ltd., Kent, UK.). Depth of anaesthesia was assessed by the absence of a flexor reflex to pinching of the hind-paw and corneal blink reflex. Rectal temperature was monitored and maintained at 37-38°C with a heated blanket and thermistor (Harvard apparatus Ltd). Two cannulae (2.5FG and 2FG; 3S healthcare, UK.) were placed in the internal jugular vein for constant anaesthetic infusion and test compound administration. The carotid artery was exposed and cannulated (3FG) with saline containing heparin (10 units ml^{-1}) for blood pressure monitoring using a real time analysis programme (Spike 2.01, Cambridge Electronic

Design, Cambridge, UK). The trachea was then cannulated (6FG; 3S healthcare), isoflurane anaesthesia discontinued and a bolus dose of sodium pentobarbitone (Saggatal, 20 $mgkg^{-1}$) given. A constant infusion of sodium pentobarbitone (Saggatal, 20 $mgkg^{-1}$ hr^{-1}) was then used to maintain anaesthesia. This anaesthetic regime had previously been found in the laboratory to produce a reliable, stable state of anaesthesia suitable for invasive surgery over periods of up to 10 hours. Animals were mechanically ventilated at a rate of between 85 and 90 cycles a minute (Harvard Apparatus Ltd. Edenbridge, Kent, UK). At the end of experiments, animals were euthanased by an intravenous injection of pentobarbital (Euthanal).

During optimisation of this method, blood gases measurements were taken at two hourly intervals over 8 hours in 10 rats and respiratory rate and tidal volume adjusted to optimise parameters using this anaesthetic protocol. It was found that ventilating with room air was sufficient to maintain blood gas reading in the range: pCO_2 35-45mmHg, pO_2 90+ mmHg, pH of 7.35-7.45 (this is within the physiological range for the anaesthetised rat). Due to problems with regular access to a blood gas analyser, in subsequent experiments, blood gas measurements were only taken at the end of an experiment.

For experiments in study 3 of this thesis, muscular paralysis was required to allow electrical stimulation of sensory nerve fibres in the face without generating muscular responses. For these experiments, the non-depolarising muscle relaxant, vecuronium bromide was administered as a constant infusion ($0.1mgkg^{-1}$ hr^{-1}) during electrophysiological recording only. Depth of anaesthesia was assessed by the absence of fluctuations in blood pressure and heart rate (using a pulse oximeter; Nonin, UK) under veterinarian supervision.

2.1.1.1 Exposure of the middle meningeal artery

The head of the rat was placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA) and a longitudinal incision made in the scalp. The parietal bone of the skull was exposed and gently thinned, using a saline-cooled drill (Foredom Electric co., Bethel, USA) to visualize a branch of the MMA. Care was taken to ensure that the bone membrane remained intact, creating a closed cranial window over the MMA. Bipolar stimulating electrodes (Clark Electromedical NEX-200) were then placed into contact with the skull over the area of the MMA to allow transcranial stimulation of the perivascular neurons innervating the MMA. The area was covered with warm mineral oil to prevent drying.

2.1.1.2 Exposure of the trigeminal nucleus caudalis

A skin incision was made from the back of the skull to the level of C_1, the overlying neck muscle was separated along the midline using blunt dissection, and held out of the way by the use of retractors. The alanto-occipital membrane and dura mater were incised to exposure the medulla and the pia mater was left intact. Due to the angle of the head, the area was continually bathed in cerebrospinal fluid hence it was not necessary to provide additional covering.

2.1.2 Intravital microscopy

The technique of intravital microscopy was developed by Shepheard *et al.* (1997) for the specific non-invasive measurement of the diameter of a particular dural vessel through a closed cranial window. Previously, laser Doppler flow measurements were used to assess meningeal blood flow as an indication of vascular changes occurring in

animal migraine models (Kurosawa *et al.*, 1995). However laser Doppler flow measurements are reliant on both blood cell velocity and concentration and hence a recorded increase in flux, cannot be assumed to be entirely contributable to vasodilatation alone. Intravital microscopy now provides a significant advance in the investigation of dural blood vessel diameter in studies of both vasodilatation and vasoconstriction and avoids many of the problems associated with an open cranial window such as brain swelling. The disadvantage of intravital microscopy is the necessity to drill the skull over the area of interest to visualise the blood vessel under study, which typically result in a constriction of the blood vessel. However, during optimisation of this technique, the MMA following visualisation was found to dilate maximally within 30 minutes after the cranial window had been formed, with no further dilatation observed during an observation period of two hours. Hence, in all experiments, the preparation was left for one hour after drilling before experimental protocols were initiated.

2.1.2.1 Data measurement

Following formation of a cranial window, the MMA was viewed under green filtered light using an intravital microscope (Moritex Europe Ltd.) attached to a computer equipped with a WinTV video capture card (Hauppage, U.K.). Dural blood vessel diameter was continuously measured using a video dimension analyser (Living Systems Instrumentation, USA) and MMA diameter, blood pressure and heart rate, were recorded using a Notocord Hem 3.3 Data Analysis system.

2.1.2.2 Analysis of data

Data was measured in arbitrary units and responses were calculated as the mean

percentage increase over resting diameter ± standard error of mean (SEM). For statistical analysis, data was analysed for significance of drug effects with a repeated measure analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test for multiple comparisons. A probability (P) value < 0.05 was considered significant.

2.1.3 Electrophysiology

In vivo electrophysiology was used for the examination of neuronal activity in the Vc resulting from manipulations of the appropriate receptive field. Current technology does not permit access directly to the peripherally located sensory receptors, hence only indirect interpretations of the events occurring at the sensory nerve ending itself could be carried out. The measurement of neuronal activity relies on an electrode that detects changes in the concentration of extracellular ions resulting from an alteration in membrane potential following the transmission of an action potential. Typically, action potentials involve a rapid alteration in membrane potential of around 100mV following an influx of charged sodium and calcium ions into the cell and an efflux of potassium ions out of the cell. These conductance alterations result in rapid membrane potential alterations that can be measured during electrophysiological experiments.

Electrodes used in this study, were made of tungsten and coated with paraffin along the length except for the tip (World Precision Instruments,). The area of the exposed tip is directly related to the electrical resistance of the electrode, with small tip electrodes having a higher resistance than larger tip electrodes as they restrict the area from which potentials can be recorded. The electrodes used for studies in this thesis, were purchased with known resistances of 2 ± 0.1 MΩ, which is considered to be a high resistance electrode and ideal for measurement of single neuronal activity.

However, the disadvantage of high resistance electrodes is the difficulty in identifying cells as the electrode has to be in extremely close proximity to the cell before a signal can be detected.

When an electrode is advanced through the Vc, the types of neuronal activity encountered include:

a) an injury response; this occurs when the electrode damages cells and results in high frequency spikes for a few seconds before the recorded activity is "lost", presumably as the cell dies.

b) spontaneously active cells; these are the most commonly observed cells and their activity can be as low as a few spikes per minute to tens of spikes per second. The majority of spontaneously active cells encountered during studies in this thesis, did not also respond to stimulation such as electrical stimulation of the MMA or mechanical stimulation of the FRF.

c) cells responding to stimulation, such as electrical stimulation of the MMA or mechanical stimulation of the FRF, which were the cell types used for this thesis.

To identify single units within the Vc, activated by either dural or facial stimulation, an electrode was lowered into the Vc by use of a micromanipulator (model FMC100, Newport Corporation, Irvine CA, USA). The electrode was then advanced through the tissue in steps of 15μm (using the electrical micromanipulator drive), while a search stimuli was applied. In some cases this was an electrical stimulus (Harvard Apparatus Ltd.) delivered to the MMA at 1Hz, 3mA and 0.3ms and in other experiments, involved light stroking of the face using a cotton tip. Identification of a cell whose

activity was linked to stimulation of the receptive field were assessed both manually using an oscilloscope and using an on-line analysis program, which is described more fully in appendix B.

The identified cell linked to stimulation was then characterised by latency of response (i.e. A-δ or C-fibre), WDR, NS or LT (see section 1.3.6.1), depth of recording and amplitude of response. The boundaries for Aδ and C-fibre responses were based on the observations that following MMA stimulation, two distinct groups of responses were seen. The first group of responses appeared 7-25 ms after stimulation, attributed to A-δ fibre input, and the second group appeared 40-80 ms after stimulation, attributed to C-fibre input. With an estimated conduction distance of 25 mm, and assuming a time of 1 ms for synaptic transmission (Eccles, 1964), a range of conduction velocities of 1.0-4.0 ms^{-1} for A-δ responses and 0.30-0.65 ms^{-1} for C-fibre responses was observed, in general agreement with standard definitions (Guyton and Hall, 1996).

2.1.3.1 Data capture

The methodology for extracellular recording of action potentials has changed very little during the past 50 years, with the exception of the technology available for the processes of data capture. These have progressed from recording nerve activity on tape and Polaroid pictures of oscilloscope traces, to the introduction of data-acquisition software which not only allows the recording of large quantities of data but also allows sophisticated analysis of the raw data. The basic recording set up used in these studies is shown diagrammatically below.

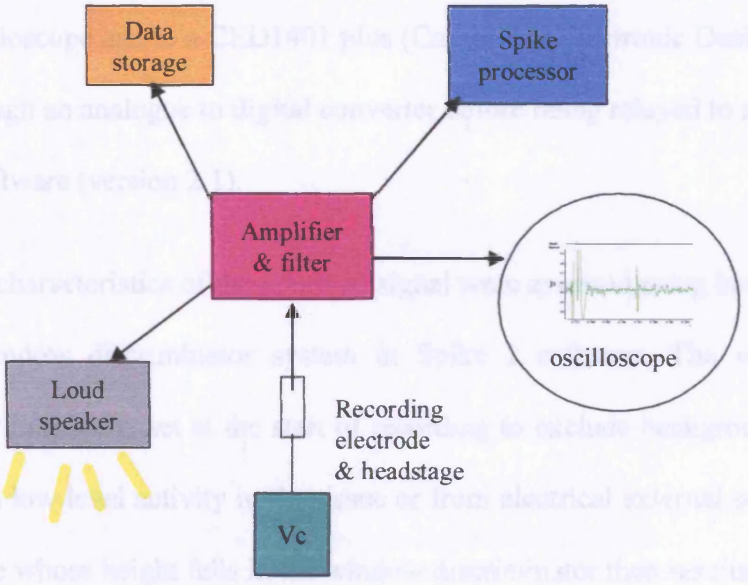


Figure 9 Electrophysiological recording system

Following the detection of a voltage change in the Vc by a tungsten electrode, the signal is amplified and filtered, processed by a PC and observed an a oscilloscope. Data is also digitally stored for any off-line analysis.

To allow experiments to be done without using a faraday cage, the system is grounded through the animal and the stereotaxic frame and differential recording allows subtraction of the signal from the animal from the neuronal signal. The signal is then amplified by the headstage (NL100AK, Neurolog, Digitimer, Herts, UK). The signal then passes to an AC-pre-amp module where the signal is split to (1.) an AC amplifier (NL104A) and (2.) a digital tape recorder for storage (Sony). If further data analysis is required at the end of an experiment, other than that obtained during on-line analysis the digital tape can be replayed and is essentially fed through the AC amplifier in the same way as the raw signal. From the AC amplifier the signal is filtered (NL125, bandwidth 500-5KHz, Neurolog.), to remove background noise and further split to the oscilloscope and to a CED1401 plus (Cambridge Electronic Design) where it is passed through an analogue to digital converter before being relayed to a computer with Spike 2 software (version 2.1).

The characteristics of the neuronal signal were assessed using both an oscilloscope and a window discriminator system in Spike 2 software. The width of the window discriminator is set at the start of recording to exclude background noise, i.e. such as from low level activity in the tissue or from electrical external source. Every neuronal spike whose height falls in the window discriminator then results in an electrical pulse, which is counted as a single event. The studies in this thesis were designed to follow the activity of a single cell. Therefore, the window was set "tightly" around the cell to exclude activity from other units in the vicinity of the electrode, such as from spontaneously active cells, or other cells that began to respond to stimulation, such as occurred during some sensitisation experiments. However, electrodes are susceptible to "drift" (i.e. small movement, estimated not to be any greater than 10μm), which

results in a decrease in the amplitude of the detected signal, possibly as a result of the animals respiratory movements or expansion of tissue following small amounts of direct heat during microscope placement of the electrode. Hence, a compromise must be made when setting the window discriminator between allowing for drift and excluding "noise" and other cells.

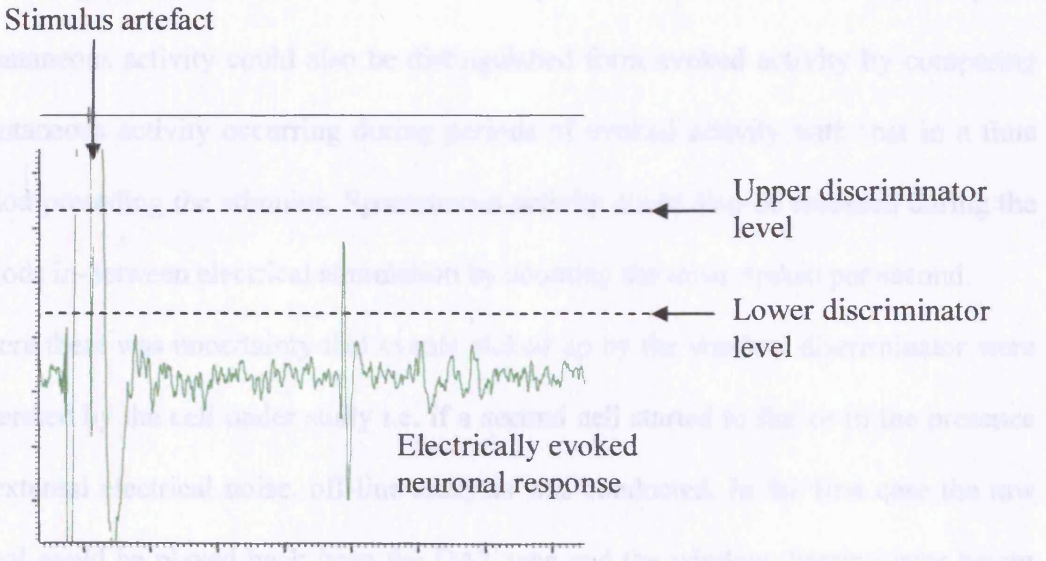


Figure 10 Window discriminator to isolate single units

The figure above shows a typical neuronal response recorded in the Vc following electrical stimulation of the MMA. Dashed lines represent the window discriminators which could be manually set to optimise recording and exclude baseline variation.

The output of the window discriminator can then be displayed in various formats in spike 2, such as post-stimulus histograms or raster plots (number of events on the Y axis and time on the x-axis). In the majority of experiments, analysis was conducted throughout the experiment using a script file created using Spike 2 software and a detailed account is given in appendix c. In brief, automated analysis was used to count the number of events occurring in a defined period after a stimulus, such that cells responding with both an A-δ and C-fibre component could be differentially analysed. Spontaneous activity could also be distinguished form evoked activity by comparing spontaneous activity occurring during periods of evoked activity with that in a time period preceding the stimulus. Spontaneous activity could also be assessed during the periods in-between electrical stimulation by counting the mean spikes per second.

Where there was uncertainty that events picked up by the window discriminator were generated by the cell under study i.e. if a second cell started to fire or in the presence of external electrical noise, off-line analysis was conducted. In the first case the raw signal could be played back from the DAT tape and the window discriminator height re-set. However if the interference still fell within the discriminator band, the raw signal was re-analysed using a waveform discrimination programme in Spike 2. The basis of this programme was the separation of different spike shapes onto different templates, the DAT was then re-played and events could be differentially analysed depending on which template they fell into. If it was still not possible to separate the events generated from the cell under test from other events, the experiment was excluded. The exclusion of data due to being unable to discriminate single units was relatively rare and in the entire course of studies in this thesis, 16 experiments were discarded on this basis. However, re-analysis was more common, especially during

PGE_2 (study 2) and GTN (study 1) experiments and approximately 1 /10 experiments was re-analysed by either resetting the height of the window discriminator or by waveform analysis.

2.1.3.2 Experimental protocols

Electrical stimulation protocols were driven from a programme designed in Spike2 software (a detailed explanation can be found in appendix B). Trains of electrical stimulation, typically 20 or 50 repetitions at 1Hz, 3 and 5 minutes apart respectively, were applied to either the dura mater or face. Baseline evoked neuronal responses were established over at least 3 trains of electrical stimulation where a variation in the evoked response of less than 10% was considered acceptable. Electrical stimulation strength was ideally set to elicit one neuronal response per stimulus (approx. 1.5 X threshold), however this was not always possible and in some cases a single stimuli resulted in 2-3 responses.

Mechanical stimulation of the FRF was also used in several experiments. Characterisation experiments of this methodology used an air jet blower, Von-Frey hairs and a cotton tip probe and serrated forceps. The disadvantage of the air jet blower in the first case was the inability to test both innocuous and noxious stimulation, furthermore, it was extremely difficult to set the pressure to a level where it only resulted in the activation of a single unit. Von-Frey hairs generally gave an inconsistent response, hence for all studies using mechanical stimulation, a cotton tip probe and serrated forceps were used to elicit responses to a light brush and pinch of the FRF. The area of the face that was most sensitive to afferent stimulation was

marked on the face by marker pen (area typically 1 cm) and the response to a light, non-noxious 10-second brush and a noxious 5-second pinch stimulation of this area, assessed.

2.1.3.3 Analysis of data

Electrophysiological data following electrical stimulation protocols is represented, where appropriate, as mean ± standard error of the mean, frequency of response or the number of spikes per second. Responses to electrical stimulation of the MMA were normalised as the total response to the train of stimulation directly before treatment and expressed as a percentage of this response. Mechanically evoked FRF evoked responses were normalised with respect to control responses and expressed as a percentage of this response. Statistical analysis was carried out as appropriate for individual studies and is described more fully in each section. In all cases, a probability (P) value < 0.05 was considered significant.

2.2 Immunohistochemistry

2.2.1 Animal preparation

It has previously been found in our laboratory that the level of *c-fos* expression in the spinal cord is increased if animals are "stressed" before the induction of general anaesthesia. Hence, to avoid any undue stress during the induction of anaesthesia, for at least a week before the start of an experiment, animals were regularly handled and habituated to the anaesthetic induction box. Furthermore, with the exception of one study, designed to replicate a previously published study, a period of two hours was

left between the induction of general anaesthesia and the start of an experimental protocol.

The strain of rat (either Sprague-Dawley or Wistar) and the anaesthetic regime used for *c-fos* expression studies are discussed in more detail in the study 1 methods section. In all studies, after the induction of anaesthesia, core temperature was maintained between 36 and 38°C by means of a rectal probe connected to a homoeothermic heating blanket controller and depth of anaesthesia was monitored by the absence of withdrawal reflex. At the end of experiments, euthanasia was performed using an overdose of sodium pentobarbitone (100mg) and perfused transcardially with 200 ml of saline containing heparin (10 units ml^{-1}) followed by 500 ml of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.3). The brains and upper cervical spinal cord were removed and post fixed in 4% paraformaldehyde for 24 hours. Brains were then placed in a cyroprotectant (30% sucrose in phosphate buffered solution) at 4 °C for 48 hours and frozen at –70°C before sectioning.

2.2.2 Tissue processing

When removed from the freezer, brains were fast frozen onto cryostat mounts in isopentane for approximately 30 seconds and then left to equibrilate at cryostat temperature of –20°C. Serial 50μm coronal sections were cut from 0.1mm rostral to area postrema to the C2 level. Every fifth section was taken for immunoprocessing (typically 45-50 sections per experiment) ensuring a systematically random sampling of tissue. Sections were placed into a 24 well tissue culture plate containing phosphate buffered saline (PBS) + sodium azide (0.01%). Remaining sections were stored at 4°C.

Immunoprocessing was carried out on free floating sections with the avidin-biotin procedure using a commercially available kit. Sections were incubated in 10% normal goat serum (NGS) in phosphate buffered saline (PBS) and 0.2% triton for 1 hour. Primary *fos* antibody (Sigma Genosys) was reconstituted in distilled water and dilutions were made in rabbit polyclonial antisera, NGS and PBS containing 0.2% triton (1:20,000 dilution) and incubated with sections for 40 – 44 hrs at 4°C. Sections were then washed thoroughly in PBS for 10 minutes at room temperature and secondary biotinylated rabbit anti-sheep antibody (Vector Laboratories, UK), diluted in immunobuffer, PBS, and NGS (1:300 dilution) added to sections for 30 minutes. Following PBS wash, sections were placed in ABC-peroxidase complex (Vector laboratories) for 30 minutes and then in a 40% solution of DAB (3,3'-diaminobenzidine tetrahydrochloride, Sigma) and hydrogen peroxide for 1 minute. Following a PBS wash, sections were mounted onto gelatin-coated slides, air-dried overnight, dehydrated by immersion in increasing concentrations of ethanol – 50%, 70%, 75%, 95%, and 100%- and cover slipped.

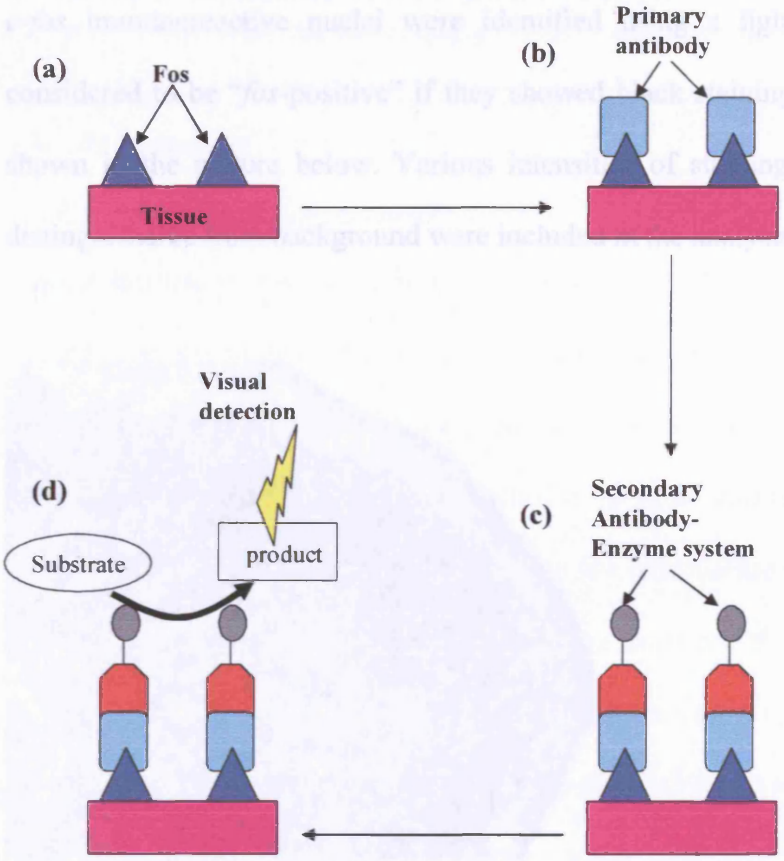


Figure 11 Labelling of *c-fos*

The above figure shows the Immunohistochemical process for the labelling of c-fos protein. a.) Following sectioning, tissue was placed in 24 well plates. b.) Fos primary antibody (extracted from the blood serum of an animal, immunised against fos protein) is incubated with the tissue. c.) This primary antibody acts as an antigen for a secondary antibody, obtained from a separate species immunised with the IgG of the primary species. The secondary antibody is biotinylated and coupled to an enzyme system. d.) avidin labelled with peroxidase is reacted with the biotin molecules. Application of DAB to the tissue then yields a reaction with the peroxidase, which results in a dark brown precipitate which can be easily identified using a microscope.

2.2.2.1 Cell counting

c-fos immunoreactive nuclei were identified using a light microscope and cells considered to be "*fos*-positive" if they showed black staining as a round structure as shown in the picture below. Various intensities of staining were observed and all distinguishable from background were included in the analysis.

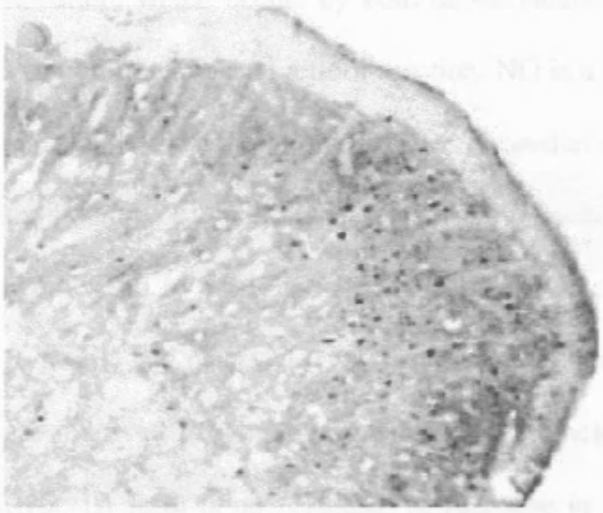


Figure 12 *c-fos* immunoreactivity in the Vc

c-fos immunoreactivity in the Vc, shown by black round staining. The intensity of staining was variable and hence all that was distinguishable from background was counted as fos-positive staining.

Sections were orientated in the rostral-caudal plane by virtue of the distinct anatomy of the sections. Using area postrema as an anatomical reference point, the location of individual sections could be identified by reference to a stereotaxic atlas (Paxinos & Watson 1998) and back calculation using the thickness of sections and number of sections to area postrema. In this manner, the profile of *c-fos* immunostaining throughout the rostro-caudal extent of the Vc + C1/C2 was established.

3 Study 1- Modulation of the trigeminovascular system with glyceryl trinitrate before and after the induction of central sensitisation

3.1 Introduction

3.1.1 Biological effects of GTN

The immediate biological effects of GTN are likely to be due to nitric oxide (NO) donation, which occurs by both an enzymatic process (glutathione-S-transferase and cytochrome P_{450}) and a thiol reaction. NO is a free radical with a short half-life and in addition to vasodilator effects, it is released at synapses and has been implicated in the modulation of peripheral and central neurotransmission. Endogenously NO is synthesised by nitric oxide synthases, of which there are three isoforms. Endothelial (type III) and neuronal (type I) nitric oxide synthases are constitutively expressed and are involved in numerous physiological functions (Yun *et al.*, 1996). The inducible (type II) form is up regulated in response to inflammatory stimuli and as such, has been suggested to be a possible mediator of the delayed migraine response following GTN infusion. Indeed, Reuter and colleagues have previously found that there is a significant increase in inducible NO synthase protein expression four hours after GTN administration in the rat meninges (Reuter *et al.*, 2001). This time course corresponds well with the time course of clinical onset of the delayed headache in migraineurs following GTN infusion.

Due to the highly lipophilic nature of both GTN and NO, both are able to easily penetrate the blood brain barrier and generate widespread central nervous system effects. Of particular relevance to migraine pathophysiology, increased *c-fos*

expression in the Vc has been demonstrated following a large systemic dose of GTN (Tassorelli & Joseph, 1995). Modulation of neuronal activity in the Vc following GTN administration, may result from several different actions. For example, GTN may directly modulate neuronal activity in the Vc, alternatively, GTN may generate inflammation in the dura mater with resultant alteration in primary afferent input into Vc. GTN administration has also been shown to modulate supraspinal sites that send descending projections to the Vc (Pardutz *et al.*, 2002; Tassorelli *et al.*, 1997).

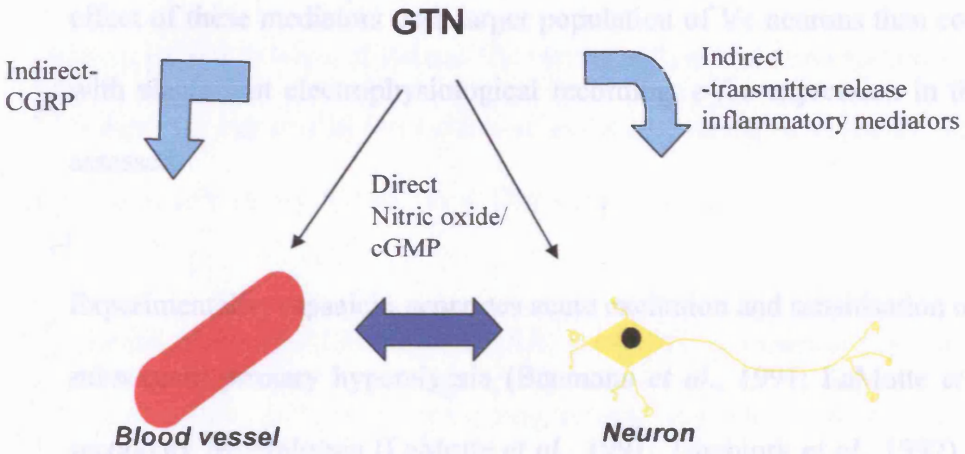


Figure 13 Possible sites of action of GTN

GTN is highly lipophilic and easily crosses the blood brain barrier. Following in vivo administration, tissue distribution has been found to be significantly higher than that in plasma, with the highest levels found in brain and heart tissue (Torfgard & Ahnler, 1991). The main bioconversion product of GTN is NO, which is able to act as a neuronal signal transmitter, resulting in both direct effects and indirect effects by causing the release of other neurotransmitters. GTN is also able to cause direct and indirect effects on blood vessels by the direct release of NO in the blood vessel wall and subsequent activation of cGMP and cGMP-dependant kinase as well as modulation of the contractile state of the vessel by activating transmitters such as CGRP, (Diagram adapted from Tassorelli et al., 1999).

3.1.2 Central sensitisation and GTN

As discussed in the introduction, the susceptibility of migraineurs to agents such as GTN or red wine may be due to a "CNS hyperexcitability", such as might occur with various ion channels pathophysiologies in brainstem structures such as the PAG or hypothalamus. Under these conditions, primary afferent activation following GTN administration may generate a greater than normal central activation. Hence, the effect of GTN infusion following the induction of central sensitisation with capsaicin, formalin, NGF and PGE_2 was explored. To generate a more complete picture of the effect of these mediators on a larger population of Vc neurons than could be obtained with single-unit electrophysiological recording, *c-fos* expression in the Vc was also assessed.

Experimentally, capsaicin generates acute excitation and sensitisation of C-fibres, with subsequent primary hyperalgesia (Baumann *et al.*, 1991; LaMotte *et al.*, 1992) and secondary hyperalgesia (LaMotte *et al.*, 1991; Torebjork *et al.*, 1992). This correlates well with studies in human volunteers, who report an intense burning pain lasting for 15-60 seconds with subsequent allodynia and hyperalgesia following capsaicin injection. Capsaicin exerts its effect through the vanilloid (VR-1) receptor, a ligand and heat gated ion channel present on small diameter sensory neurons (Caterina *et al.*, 1997). The presence of VR-1 has been demonstrated on the cell bodies of trigeminal afferents in the rat trigeminal ganglion (reported ranges vary from 14 to 26% of cell bodies; Ichikawa & Sugimoto, 2000 & 2001) and also in laminae I and II of the Vc.

Peripheral formalin injection produces a long-lasting nociceptive response in animals and as such, formalin injection is widely used as a model of pain for the investigation of novel pain drugs. The formalin pain model has two distinct phases with an acute pain behavioural response seen 3-5 minutes after formalin injection, followed by a quiescent period of 10-15 minutes. The second pain behavioural response starts 15-20 minutes after formalin injection and lasts for a further 20 to 40 minutes (Lebrun *et al.*, 2000). This biphasic nature can also be observed using electrophysiological recording following the injection of formalin into the receptive field (Puig & Sorkin, 1995). The initial phase is directly attributable to activation of sensory afferents while the second phase is the subject of debate. However, differential time-related roles of different transmitter systems in the induction and maintenance of formalin induced pain have been clearly shown (Chapman & Dickenson, 1995).

The high-affinity NGF receptor, trkA, is selectively expressed by nociceptive sensory neurons particularly those containing sensory neuropeptides such as substance P and CGRP (McMahon, 1996). Furthermore, recent studies have suggested that NGF is a peripherally produced mediator of some persistent pain states, notably those associated with inflammation and systematic or local application of exogenous NGF produces a rapid and prolonged behavioural hyperalgesia in both animals and humans (Mendell *et al.*, 1999; Tal, 1999, Bennett, 2000). Additionally, some work carried out in our laboratory suggested that NGF has a sensitising effect of cultured trigeminal neurons (Jenkins *et al.*, personal communication). The rationale for the use of PGE_2 to induce sensitisation is discussed more fully in the introduction to study two, but was based on the known effectiveness of NSAIDs in the treatment of acute migraine pain indicating a prostaglandin-mediated sensitisation may be responsible for at least a component of

the hypersensitivity seen during migraine (Diener *et al.*, 1999).

3.2 Methods

3.2.1 Intra-arterial infusions and topical administration of GTN

Electrophysiological recordings and intravital microscopy were carried out as described in section 2.1. For experiments involving intra-arterial GTN infusions, a second cannula was placed rostrally in the same carotid artery cannulated for blood pressure monitoring, for GTN infusion to the cerebral circulation.

3.2.1.1 Intravital microscopy

Due to the technical difficulty of this intravital microscopy, the protocol was designed to minimise the number of experiments performed, hence GTN vehicle and different GTN doses were tested in a sequential manner in the same animal. Following establishment of a steady baseline diameter of the MMA (over at least fifteen minutes) an experimental protocol of GTN vehicle (glucose-saline) intra-arterial infusion followed by GTN intra-arterial infusions at 2, 5 and 10$\mu gkg^{-1}min^{-1}$ was started. Infusions were sequential and each dose of GTN and GTN vehicle were infused for twenty minutes with a five-minute gap left between doses or, where a response was seen, five minutes after return of MMA diameter to pre-treatment baseline values. After infusion of GTN (10$\mu gkg^{-1}min^{-1}$), a bolus intravenous dose of CGRP was administered (300$ngkg^{-1}$) for comparison with data previously obtained in the laboratory (*A. Honey, unpublished data*). The sequential infusions were then repeated i.e. GTN vehicle for twenty minutes followed by GTN infusion 2, 5 and 10$\mu gkg^{-1}min^{-1}$ for twenty minutes each.

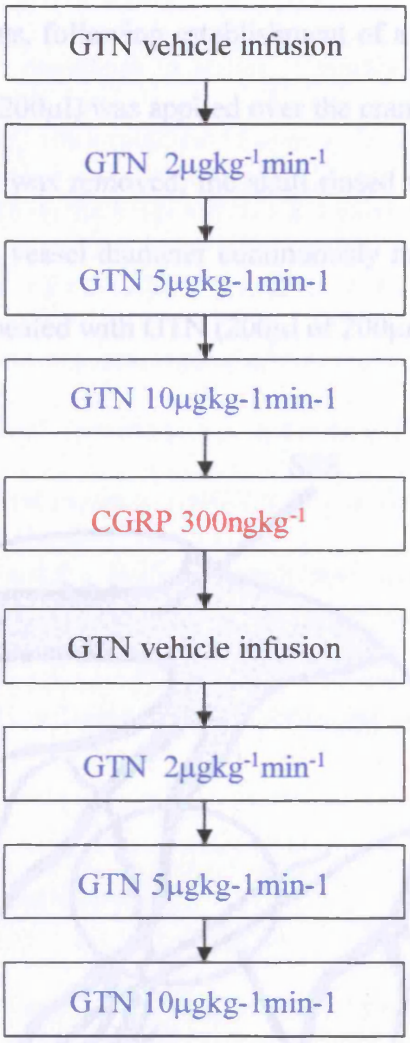


Figure 14 Intravital microscopy-experimental protocol

For experiments examining the effect of intra-arterial GTN infusion using intravital microscopy, sequential infusions (20 minutes each) were examined with a 5 minute gap left between infusions or where a response was seen, the time for vessel diameter to return to normal plus 5 minutes. Following infusion of GTN vehicle, and GTN at 2, 5 and 10μgkg^{-1}, a bolus dose of CGRP (300ngkg^{-1}) was given intravenously and the GTN vehicle and GTN infusions were repeated.

In separate experiments, following establishment of a steady baseline diameter of the MMA, GTN vehicle (200µl) was applied over the cranial window on a gauze swab for 5 minutes. The gauze was removed, the skull rinsed with saline and re-covered with mineral oil and blood vessel diameter continuously monitored. After fifteen minutes, this procedure was repeated with GTN (200µl of 200µmol solution).

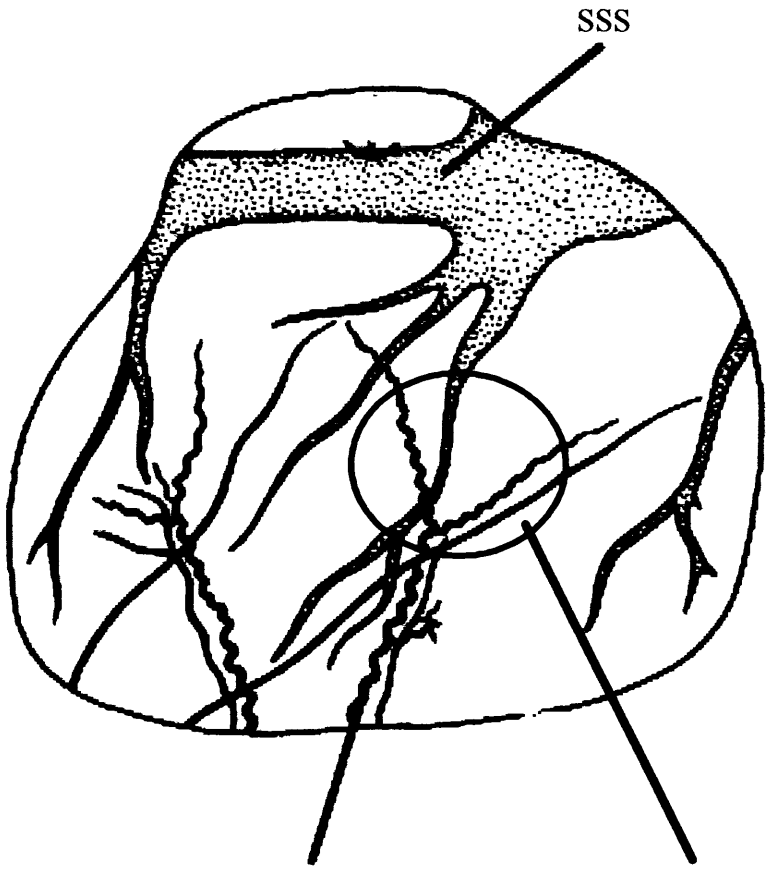


Figure 15 Topical application of GTN over the closed cranial window

For topical application of GTN or GTN vehicle, a gauze swab was saturated with the appropriate solution and placed over a region of the MMA that could be visualised under a microscope following thinning of the skull to form a closed cranial window. (Diagram adapted from Ellrich et al., 1999).

3.2.1.2 Electrophysiology

Reproducible baseline responses to trains of electrical stimulation (50 stimulations, every 5 minutes at 1Hz) were established over at least fifteen minutes where less than 10% variation was seen in the response to successive trains. Intra-arterial infusions of GTN ($5\mu gkgmin^{-1}$) or vehicle (glucose-saline) were then administered over twenty minutes. In separate experiments, topical administration of GTN vehicle and GTN was carried out as described for intravital microscopy. Electrophysiological data was analysed using a repeated measure ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons and $P < 0.05$ was considered significant.

3.2.2 Intravenous infusions of GTN following induction of central sensitisation

3.2.2.1 Electrophysiology

Baseline electrophysiological recording of second order neurons in the Vc responding to electrical stimulation of the MMA with Aδ- latency were carried out as described above.

Formalin (0.3 or 0.5 or 1 or 2% in 50µl of saline) or capsaicin (3mM) or vehicle (formalin vehicle -saline; capsaicin vehicle-10% ethanol, 10% Tween 80 in saline) were administered adjacent to the FRF of the test Vc neuron. The effects of capsaicin application directly to the cranial window overlying the MMA were also tested. In these experiments, capsaicin (3mM) was applied over the cranial window on a gauze swab for a period of 2 minutes before the area was washed with saline and re-covered with mineral oil. In separate experiments, topical capsaicin application, the effects of

intravenous GTN infusion (2 or 5μgkg^{-1}min^{-1} over 30 minutes) was assessed. In the experiments, GTN or GTN vehicle infusion was started 10 minutes after capsaicin administration and continued for 30 minutes.

As both formalin and capsaicin directly activate primary afferent neurons, in this study both mediators were administered adjacent to the receptive field to avoid directly altering the characteristics of the cell under study. Hence, any effect on neuronal activity could be assumed to result from central changes, most likely occurring through the process of "heterosynaptic facilitation" (Thompson *et al.*, 1993).

3.2.3 Immunohistochemistry

For all studies in this section, immunoprocessing of tissue was carried out as described in section 2.2. Except were stated, male Sprague-Dawley rats (300-430g) were anaesthetised using 5% isoflurane in oxygen. An angiocath (interocath 22G) was inserted in a tail vein (patency was ensured with regular 0.1ml flushes of heparinised saline, 10 unit/ml). α-Chloralose (80 mg/kg i.v.) was administered as a slow bolus (over 2 minutes), isoflurane anaesthesia gradually reduced and animals allowed to respire spontaneously. Further doses of α-chloralose (20 mg/kg i.v. or 40mg/kg i.v. depending on the duration of the experiment) were given at approximately hourly intervals.

3.2.3.1 Experimental protocol using formalin as a mediator of central sensitisation

Following a two-hour stabilisation period (measured from the time of first

administration of α-chloralose), formalin (0.5% or 5% in 50μl) or vehicle (saline) was injected into the maxillary or infraorbital region of the face. Ten minutes later, GTN ($5\mu gkg^{-1}min^{-1}$) or vehicle (glucose saline) was infused into the tail vein for 30 minutes. Either two or four hours after GTN infusion, euthanasia was performed via a pentobarbital overdose.

3.2.3.2 Experimental protocol using capsaicin as a mediator of central sensitisation

In the first instance, animals were subjected to a two-hour stabilisation period following administration of α-chloralose, as described for formalin. GTN ($2\ \mu gkg^{-1}\ min^{-1}$) or vehicle (glucose saline) was then infused into the tail vein for 30 minutes. Immediately following the infusion period, a 50μl bolus dose of capsaicin (0.02%) or vehicle (10% ethanol, 10% Tween 80 and saline) was injected intradermally into the infraorbital region of the face, using a Hamilton syringe. One-and-a-half hours after the end of the GTN infusion, euthanasia was performed via a pentobarbital overdose.

During the course of these studies, reports examining an almost identical protocol using a combination of capsaicin and GTN were published (Jones *et al.*, 2001). The authors of this study reported that intradermal injection of capsaicin injection into the periorbital area increased *c-fos* expression in the Vc and furthermore, GTN infusion facilitated the expression compared to vehicle infusion. Hence, to allow a comparison between methodologies, the experimental conditions of this paper were replicated as part of this thesis. Briefly, male Wistar rats (200-250g) were anaesthetised with an intraperitoneal injection of urethane (1.5g/kg). The left femoral vein was cannulated

and GTN (2-2.5 μgkg^{-1} min^{-1}) or GTN vehicle was infused for 30min, without any stabilisation period, following surgery. Immediately following the infusion period, a 50µl bolus dose of capsaicin (0.02%) or vehicle (10% ethanol, 10% Tween 80 and saline) was injected intradermally into the infraorbital region of the face. One-and-a-half hours after the end of infusion, euthanasia was performed via a pentobarbital overdose.

3.2.3.3 Experimental protocol using Prostaglandin E_2 (PGE_2) and Nerve Growth Factor (NGF) as a mediator of central sensitisation

As described for capsaicin and formalin above, following induction of anesthesia with α-chloralose, animals were given a two hour stabilization period. The following combination of drugs were then injected intradermally into the infraorbital region of the face as two separate injections immediately following each other.

i.) NGF (2.5µg in 50µl) and PGE_2 (2µg in 50µl)

ii.) NGF vehicle (saline, 50µl) and PGE_2 (2µg in 50µl)

iii.) NGF (2.5µg in 50µl) and PGE_2 vehicle (saline, 50µl)

Ten minutes after injection of the inflammatory mediator, GTN (5μgkg^{-1} min^{-1}) or GTN vehicle was infused through the tail vein for 30 minutes Two hours after GTN infusion, euthanasia was performed via a pentobarbital overdose.

3.2.3.4 Statistical analysis

Data from all immunohistochemistry experiments were considered in terms of both

total *c-fos* expression in all sections processed and mean *c-fos* expression per section. Tissue from each complete study, usually consisting of the following 4 groups, was processed together, i.e. all reactants for a particular study were from the same batch, hence allowing an accurate comparison within a study group.

i.) inflammatory mediator vehicle & GTN vehicle

ii.) inflammatory mediator vehicle & GTN

iii.) inflammatory mediator & GTN vehicle

iv.) inflammatory mediator & GTN

A considerable variation in baseline *c-fos* expression was observed between study groups, even for identical treatments e.g. inflammatory mediator vehicle & GTN vehicle, which may in part be due to tissue processing rather than experimental variation. Hence, these studies were limited to comparisons of raw data within groups and the magnitude of any increased effect between study groups. Data was analysed using a repeated measures ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons and $P < 0.05$ was considered significant.

3.3 Results

Data from electrophysiological and intravital experiments included in this study was from animals with acceptable respiratory and cardiovascular parameters for the anaesthetised rat and was in the range: pCO_2 35-45mmHg, pO_2 90+ mmHg and a pH of 7.35-7.45. However, respiratory and cardiovascular parameters were not assessed in animals used for the immunohistochemistry section due to the non-invasive nature of the experimental protocol.

Data from this study in considered in the following sub-groups;

Intravital Microscopy

Intra-arterial infusion

Topical GTN administration

Electrophysiology

Intra-arterial GTN infusions

Topical GTN application over the MMA

Intravenous GTN infusions-

Using Formalin to induce CS

Using Capsaicin to induce CS

Immunohistochemistry with intra-venous GTN infusion

Using Formalin to induce CS

Using Capsaicin to induce CS

Using PGE_2 and NGF to induce CS

3.3.1 Intravital Microscopy

Mean arterial blood pressure (MABP) was not significantly affected by infusions of GTN up to doses of $5\mu gkg^{-1}min^{-1}$ however, infusion of $10\mu gkg^{-1}min^{-1}$ resulted in a depression of MABP of 38 ± 6 mmHg (n = 3), evident 6 to 9 minutes after the start of the infusion. Topical administration of GTN also significantly depressed MABP by 27 ± 5 mmHg (n = 7) within 20 seconds of application. GTN vehicle had no significant effect on cardiovascular parameters in any experiments ($P > 0.05$).

3.3.1.1 Intra-arterial infusion

Sequential infusions of GTN vehicle, GTN (2, 5, or $10\mu gkg^{-1}min^{-1}$) were carried out for 20 minutes with 5 minutes between infusion where no effect on MMA diameter was seen or where an effect was seen, the time to return to baseline diameter plus 5 minutes. No effect on MMA diameter was seen following infusions of GTN vehicle or GTN (2 or $5\mu gkg^{-1}min^{-1}$; n = 3). However following infusion of GTN ($10\mu gkg^{-1}min^{-1}$) an increase in MMA diameter of 58 ± 10% was seen (n = 3). Following return of MMA to baseline levels, CGRP ($300ngkg^{-1}$) was then administered and resulted in a dilatation of the MMA of 152 ± 11% above resting diameter (n = 3). GTN vehicle and GTN (2, 5, or $10\mu gkg^{-1}min^{-1}$) infusions were then repeated following vessel normalisation. Here, GTN vehicle and GTN infusion ($2\mu g\ kg^{-1}min^{-1}$) again had no effect on blood vessel diameter (n = 3), however GTN ($5\mu g\ kg^{-1}min^{-1}$) resulted in a dilatation of the MMA of 33 ± 10% above baseline (n = 3). GTN ($10\mu g\ kg^{-1}min^{-1}$) again generated a dilatation of the MMA of 76 ± 18% (n = 3). The low numbers in this

study do not permit statistical analysis and ideally another 2 to 3 animals would have been included in the group, however, due damage to the equipment, this was not possible during the time course of this thesis.

Time	Stage of experiment	Effect
0 minutes	Baseline determination	-
15 minutes	GTN vehicle	No effect on MMA diameter (n = 3)
40 minutes	GTN -2μgkg^{-1}min^{-1}	No effect on MMA diameter (n = 3)
65 minutes	GTN -5μgkg^{-1}min^{-1}	No effect on MMA diameter (n = 3)
90 minutes	GTN -10μgkg^{-1}min^{-1}	↑ of 58 ± 10% above resting diameter (64 ± 12 seconds after the start of infusion (n = 3). MMA diameter returned to baseline levels 118 ± 19 seconds after the end of infusion.
117 minutes	CGRP	↑ of 152 ± 11% above resting diameter (16 ± 3 seconds after the start of infusion (n = 3). MMA diameter returned to baseline levels 288 ± 27 seconds after administration.
127 minutes	GTN vehicle	No effect on MMA diameter (n = 3)
152 minutes	GTN -2μgkg^{-1}min^{-1}	No effect on MMA diameter (n = 3)
177 minutes	GTN -5μgkg^{-1}min^{-1}	↑ of 33 ± 10% above resting diameter (76 ± 21 seconds after the start of infusion (n = 3). MMA diameter started to return to baseline levels during the infusion and in all cases had returned to baseline levels by the end of infusion
202 minutes	GTN -10μgkg^{-1}min^{-1}	↑ of 76 ± 18% above resting diameter (32 ± 9 seconds after the start of infusion (n = 3). MMA diameter returned to baseline levels 206 ± 33 seconds after administration.

Table 5 Summary of the effects of GTN infusion on MMA diameter

3.3.1.2 Topical GTN administration

In these experiments, direct application of GTN ($200\mu mol^{-1}$) to the cranial window, as described in section 3.2.1.1. was examined. Topical application of GTN or GTN vehicle to the cranial window disrupted the initial signal following application, obscuring the first few seconds of the response; hence, the onset of action of GTN in resulting in dilatation of the MMA in these experiments cannot be accurately assessed. However, once the gauze containing GTN was in place, a maximum dilatation of the MMA of 142 ± 20% (n = 3) above baseline diameter (n = 3) could be observed. The dilatation persisted throughout GTN application (5 minutes) and MMA diameter returned to baseline level 256 ± 35 seconds after the gauze was removed. Application of GTN vehicle to the cranial window also resulted in an increase in MMA diameter over baseline of 28 ± 22% (n = 3). However, MMA diameter returned to baseline during application (95 ± 16 seconds after gauze application) suggesting that the small dilatation may be due to the process of mechanical stimulation when the gauze was put in place.

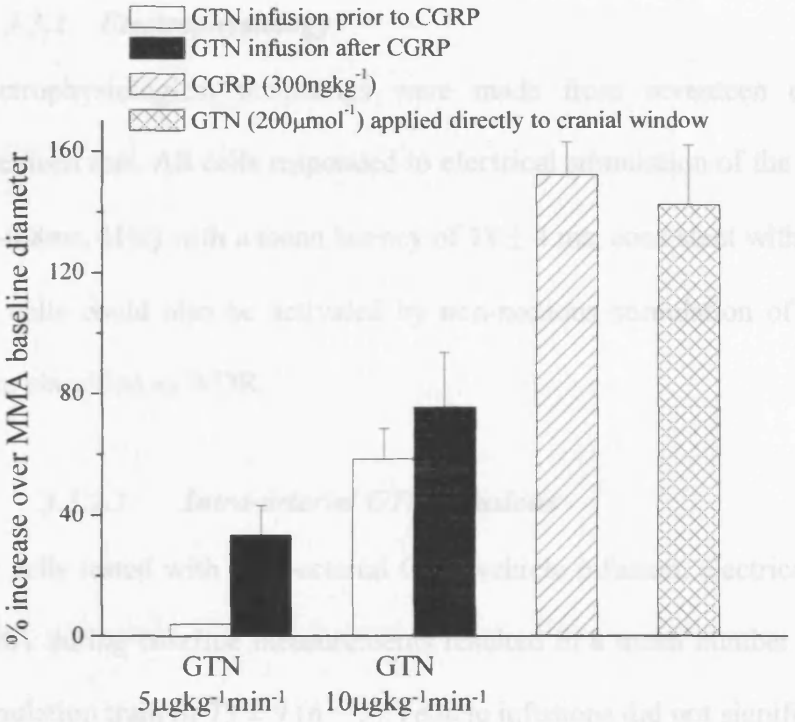


Figure 16 Dilatation of the MMA following GTN

Data is represented as the mean maximum percentage increase over resting diameter, assessed by intravital microscopy following GTN intra-arterial infusion, CGRP administration and topical application of GTN to the area overlying the MMA. Following GTN infusion (5μgkg^{-1}min^{-1}), no effect was seen on MMA diameter (n = 3), however GTN infusion (10μgkg^{-1}min^{-1}) resulted in a dilatation of the MMA of 58 ± 10%; n = 3). In the same animals, a bolus dose of CGRP (300ngkg^{-1}) resulted in a dilation of MMA of 152 ± 11% (n = 3) above resting diameter and GTN infusions were then repeated. Here, GTN infusion (5μgkg^{-1}min^{-1}) resulted in a dilatation of the MMA of 33 ± 10% above baseline (n = 3) and GTN (10μg kg^{-1}min^{-1}) generated a dilatation of the MMA of 76 ± 18% (n = 3). In separate animals GTN application (200μmol^{-1}) to the area overlying the MMA resulted in a dilatation of the MMA of 142 ± 20% (n = 3).

3.3.2 Electrophysiology

Electrophysiological recordings were made from seventeen cells in the Vc of seventeen rats. All cells responded to electrical stimulation of the dura mater (1-6mA, 0.1-0.8ms, 1Hz) with a mean latency of 18 ± 4 ms, consistent with Aδ fibre responses. All cells could also be activated by non-noxious stimulation of the face and hence were classified as WDR.

3.3.2.1 Intra-arterial GTN infusions

For cells tested with intra-arterial GTN vehicle infusion, electrical stimulation of the MMA during baseline measurements resulted in a mean number of responses to one stimulation train of 73 ± 9 (n = 5).Vehicle infusions did not significantly alter baseline recordings, at any time point during the experiment in any cell tested ($P > 0.05$). For cells tested with intra-arterial GTN infusion, electrical stimulation of the MMA during baseline measurements resulted in a mean number of responses to trains of fifty electrical stimulations of 66 ± 13 (n = 6). Following GTN infusion ($5\mu gkg^{-1}min^{-1}$), 3/6 cells showed an increased firing rate above baseline and 3/6 cells showed an inhibition of MMA-evoked neuronal activity. When considered together, the mean response of all 6 cells showed a significant decrease in neuronal activity compared to both baseline measurements and GTN vehicle infusion ($P < 0.05$) and data is summarised in the table below.

	GTN vehicle		GTN 5μgkg^{-1}min^{-1}	
	Baseline	Infusion	Baseline	Infusion
Mean ± SEM	1.0 ± 0.01	1.0 ± 0.02	1.0 ± 1.0	0.9 ± 0.08
Range of normalised values	0.24	0.68	0.26	2.79

Table 6 Effects of GTN on MMA-evoked, Vc responses

The above table gives the mean normalised recordings and the normalised data range, prior to GTN or GTN vehicle infusion and during the infusion.

Four of six cells tested with intra-arterial GTN infusion were spontaneously active with mean ongoing rates of activity of 0.5, 0.8, 0.9 and 1.6 spikes per second during the pre-treatment period. Following intra-arterial GTN infusion spontaneous activity was inhibited to a maximum of 58 ± 12% (n = 4) below pre-treatment values.

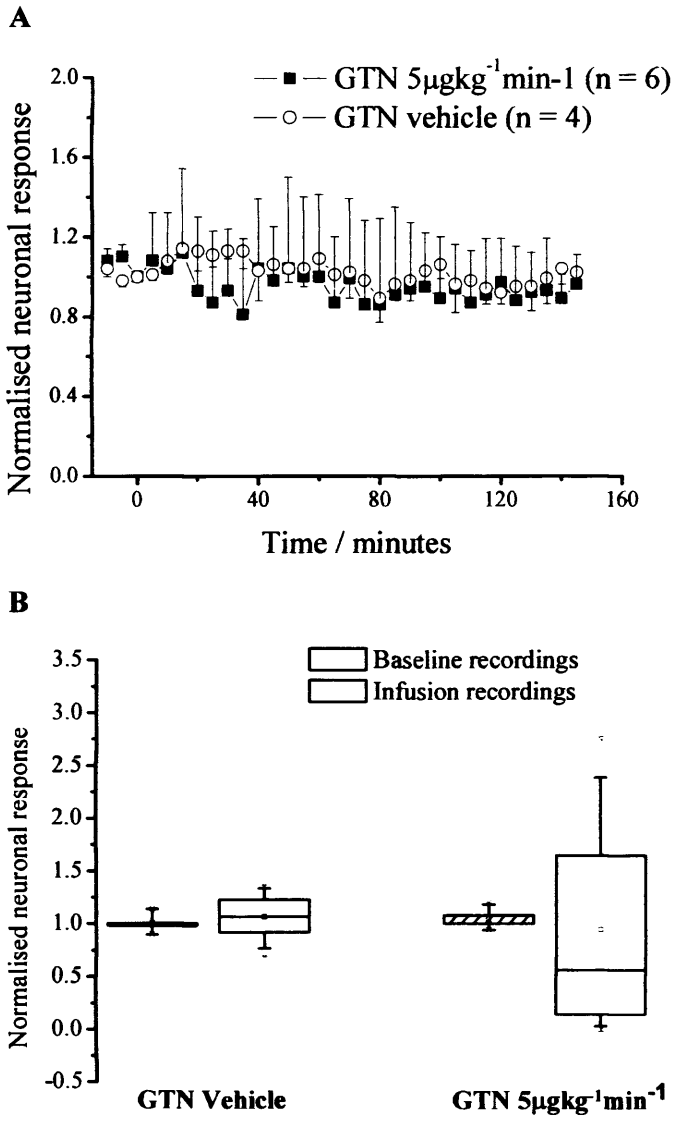


Figure 17 Effect of intra-arterial GTN infusion on evoked activity

Electrophysiological recordings were made as described in section 2.1-2.2. A. Data is represented as the mean normalised response to trains of fifty electrical stimuli applied to the MMA, recorded every five minutes. Error bars for GTN infusions are shown in the "plus" direction, and for GTN vehicle, in the" minus" direction. GTN or vehicle were infused between 0 and 20 minutes. B. Box-whisker plots show the range of the normalised responses recorded prior to GTN vehicle infusion (n =12 measurements from 4 animals), during and after GTN vehicle infusion (n =92 measurements from 4 animals), prior to GTN infusion (n =20 measurements from 6 animals), during and after GTN vehicle infusion (n =174 measurements from 6 animals). GTN resulted in a significant decrease in evoked neuronal activity compared to baseline and to GTN vehicle infusion ($P < 0.05$, ANOVA followed by Bonferroni's post hoc test for multiple comparisons), whereas GTN vehicle had no significant effect on neuronal activity compared to baseline measurements ($P > 0.05$).

3.3.2.2 Topical GTN application over the MMA

For cells tested with topical GTN application over the MMA, electrical stimulation of the MMA during baseline measurements resulted in a mean number of responses to trains of fifty electrical stimulations of 59 ± 9 (n = 4). The overall effect of topical GTN application (200µl of 200µmol solution) on MMA-evoked Vc firing was not significantly different to baseline measurements ($P > 0.05$). However, when the response of individual experiments were considered two out of four cells showed an increased response and two cells showed a decrease to MMA stimulation above baseline.

For cells tested with topical GTN vehicle administration, electrical stimulation of the MMA during baseline measurements resulted in a mean number of responses to trains of fifty electrical stimulations of 71 ± 6 (n = 3). Topical vehicle application resulted in a significant increase to MMA-evoked firing of 24 ± 2% (mean number of response to one stimulation train of 88 ± 3; n = 3; $P < 0.05$) over pre-treatment evoked firing, five minutes after application that had returned to pre-treatment levels 10 minutes after application.

3.3.3 Intravenous GTN infusions

3.3.3.1 Formalin induced sensitisation

Mean arterial blood pressure (MABP) was not significantly affected by formalin injection up to 1%, however; injection of formalin, 2 and 5% resulted in an elevation of MABP of 15 ± 2mmHg and 22 ± 5mmHg, respectively. Electrophysiological

recordings were made in 26 cells in 26 rats. All cells responded to dural stimulation in the range consistent with A-δ fibre mediated responses with a mean latency of 13 ± 3ms (range; 9-21ms). All cells also received a convergent input from the face following non-noxious stimulation and were hence, classified as WDR. In the first instance, a low dose of formalin (0.3%) was used to investigate formalin-induced sensitisation, as no sensitisation was seen at this dose, the concentration of formalin was increased incrementally to 0.5, 1 and 2%. Formalin or formalin vehicle followed by either GTN or GTN vehicle infusion did not produce any sensitisation of the evoked response to MMA stimulation at any dose tested. While the numbers in this study were low and we cannot rule out the possibility that with more experiments, a sensitising effect of formalin would be seen, the purpose of the experiment was to look at the effects of GTN following a robust sensitisation at the level of the Vc and not at the effect of formalin *per se.,* hence no attempt was made to increase the numbers in this study and capsaicin was explored as an alternative inducer of central sensitization.

	Rats	Mean cell depth (um)	Max increase over baseline responses (%)	Comment
Formalin vehicle & GTN 2μgkg^{-1} min^{-1}	3	1025 ± 115	8 ± 5	
Formalin vehicle & GTN 5μgkg^{-1} min^{-1}	2	968	18	
Formalin 0.3% & GTN 5μgkg^{-1} min^{-1}	2	1011	5	
Formalin 0.5% & GTN 5μgkg^{-1} min^{-1}	3	870 ± 119	17 ± 6	
Formalin 0.5% & GTN vehicle	3	804 ± 108	14 ± 3	
Formalin 1% & GTN 5μgkg^{-1} min^{-1}	3	862 ± 89	27 ± 25	1 cell "lost" after 15 minutes
Formalin 1% & GTN vehicle	3	671 ± 92	32 ± 13	
Formalin 2% & GTN 5μgkg^{-1} min^{-1}	2	982	-	Cells "lost" 5 and 15 minutes after formalin
Formalin 2% & GTN vehicle	3	1029 ± 238	17 ± 13	

Table 7 Summary of the maximum responses seen following formalin & GTN

Administration of 5% formalin around the FRF was also examined and while no sensitisation of cells responding to MMA stimulation could be observed, pressor effects on blood pressure, that were unrelated to depth of anaesthesia, can be seen in response to electrical stimulation of the MMA. While this could be a non-specific effect, it may indicate the development of generalised central hypersensitivity.

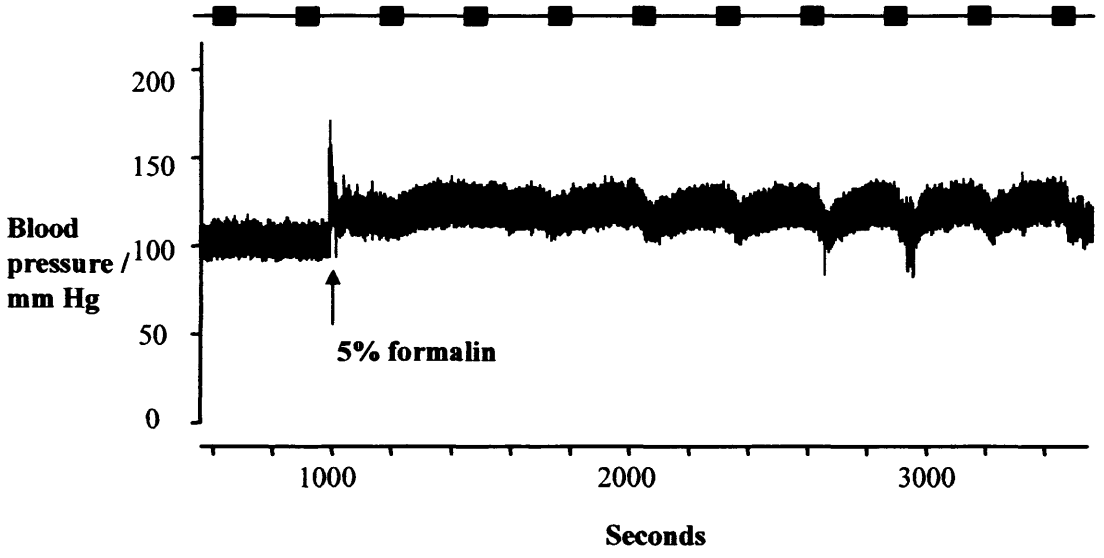


Figure 18 Formalin (5%) into the FRF generates pressor effects on blood pressure

Trace shows blood pressure recording during an electrophysiology experiment where 5% formalin was administered into the FRF (marked by arrow). Black blocks at the top of the trace show where trains of electrical stimuli applied to the MMA. Fifteen minutes after formalin administration, pressor effects on blood pressure were evident possibly indicating the development of generalised central hypersensitivity.

3.3.3.2 Capsaicin induced sensitisation

Electrophysiological recordings were made from 20 cells in the Vc of 20 rats responding to electrical stimulation of the MMA with a mean latency of 15 ± 2ms (range; 8-25ms). Cells were located in the depth range 415-1550μm below the pial surface. Mean arterial blood pressure (MABP) was not significantly affected by capsaicin.

3.3.3.2.1 Topical capsaicin application over the MMA

For cells tested with topical application of capsaicin (3mM) over the MMA, electrical stimulation of the MMA during baseline measurements resulted in a mean number of responses to one electrical stimulation train of 59 ± 8 (n = 5). Following capsaicin application, an increase in the evoked response to MMA stimulation occurred which reached a maximum of 48 ± 15% (mean number of response to one stimulation train of 79 ± 9; n = 5; $P < 0.05$) over pre-treatment values, 35 minutes after application. Electrical stimulation of the MMA during baseline measurements for cells tested with capsaicin vehicle (10% ethanol, 10% Tween 80 in saline) resulted in a mean number of responses to one electrical stimulation train of 71 ± 14 (n = 4). This firing rate was not altered from pre-treatment levels at after capsaicin vehicle at any time point during the experiment (n = 3).

3.3.3.2.2 Topical capsaicin application over the MMA and GTN

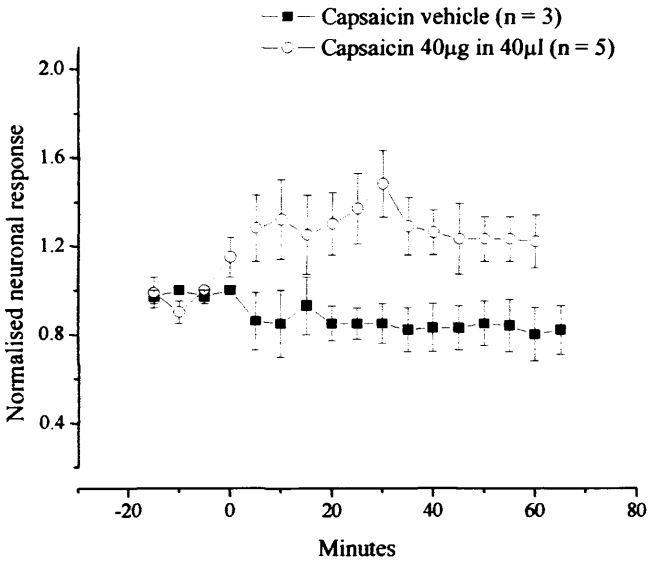
In separate experiments, topical application of capsaicin (3mM) over the MMA was

followed by infusion of either GTN vehicle or GTN infusion ($5ugkg^{-1}min^{-1}$). Electrical stimulation of the MMA during baseline measurements in these studies resulted in a mean number of responses to one electrical stimulation train of 63 ± 11 (n = 6). However, the mean maximum increase over pre-treatment levels seen following capsaicin and GTN infusion (35 ± 9%; n = 3) was not greater than the maximum increase seen following capsaicin and GTN vehicle infusion (39 ± 15 %; n = 3).

3.3.3.2.3 Injection of capsaicin in the FRF

Baseline responses to electrical stimulation of the MMA in experiments where capsaicin was injected into the area adjacent to the FRF resulted in a mean number of responses to one electrical stimulation train of 73 ± 10 (n = 3). Following sub-cutaneous injection of capsaicin into a region just outside the FRF, an increase in the response to electrical stimulation of the MMA occurred 20 minutes after capsaicin administration compared to vehicle and pre-treatment values (n = 3).

A Topical Capsaicin application over the MMA



B Capsaicin administration into the FRF

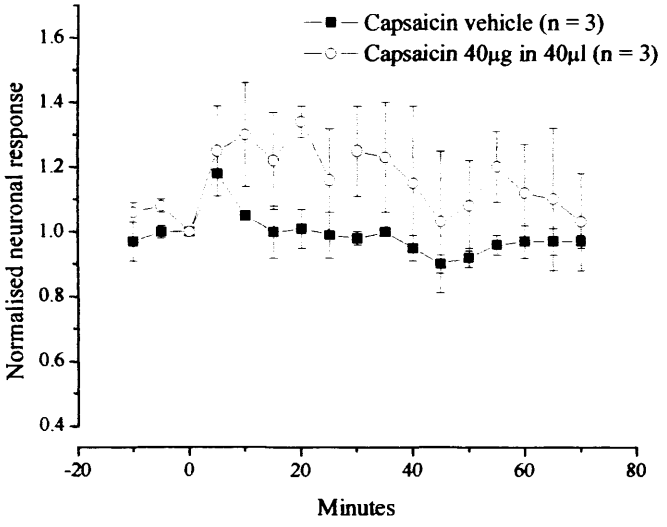


Figure 19 Mean normalised evoked responses following capsaicin

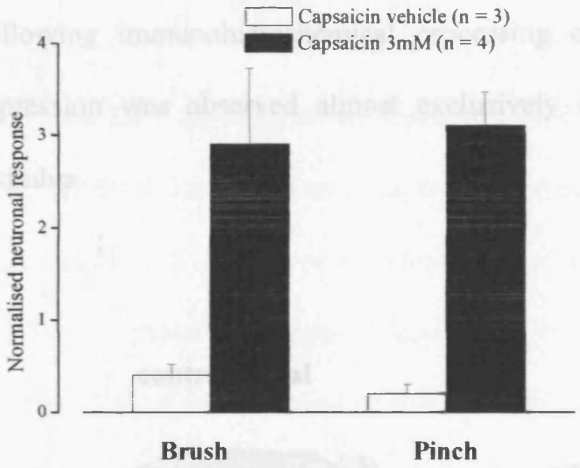
Electrophysiological recordings were made as described in section 2.1-2.2. Data is represented as the mean normalised response to trains of fifty electrical stimuli applied to the MMA, recorded every five minutes. Capsaicin was given at time 0, over the MMA (A) and into the FRF (B). Application of capsaicin over the MMA resulted in a sensitisation of 48 ± 15% (n = 5) that remained for the duration of the experiment whereas capsaicin administration around the FRF resulted in a lesser sensitisation that occurred 20 minutes after administration and returned to pre-treatment levels by the next stimulation train.

3.3.3.2.4 The effect of capsaicin on FRF stimulation

All cells tested with capsaicin or capsaicin vehicle had a FRF that typically extended over an area of the face innervated by the first or second division of the trigeminal nerve. The mean number of spikes resulting from brush and pinch stimulation of the FRF was variable between cells with a range for pre-treatment brush stimulation of 29-114 spikes per 10 second stimulus and a range for pre-treatment pinch stimulation of 37-180 spikes per 5 second stimulus, hence, data is expressed as the normalised increase over pre-treatment responses. Application of capsaicin vehicle to the MMA resulted in an increased response to brush and pinch stimulation of 40 ± 12% and 23 ± 10% respectively (n = 4; $P < 0.05$) over pre-treatment values. However capsaicin (3mM) application over the MMA produced a much larger increase to brush and pinch stimulation than that of vehicle of 300 ± 81% and 310 ± 35% (n = 4; $P < 0.05$) respectively. The maximum responses seen for both capsaicin and vehicle occurred 30 minutes after application.

The response to brush and pinch stimulation of the FRF following capsaicin administration into the area surrounding the FRF was also significantly increased, 30 minutes after capsaicin application. The mean maximum response to brush and pinch was 101 ± 21% and 30 ± 13% respectively (n = 3; $P < 0.05$) which was significantly greater than the increase seen following application of capsaicin vehicle, where the mean maximum response to brush and pinch was 10 ± 9% and 6 ± 4% respectively (n = 3; $P > 0.05$).

(a) Capsaicin application over the MMA



(b) Capsaicin administration into the FRF

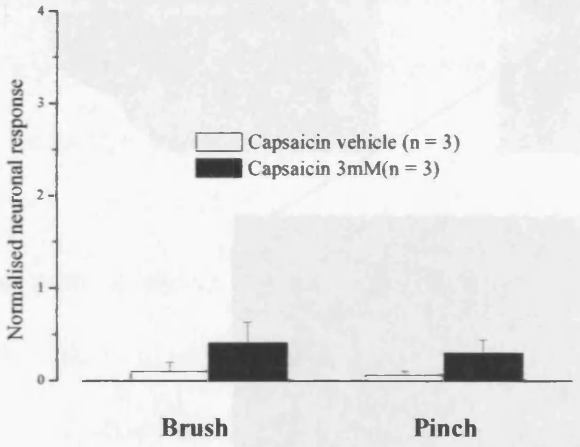


Figure 20 Response to facial receptive field stimulation following capsaicin

Electrophysiological responses to a 10-second brush and 5-second pinch were assessed 3 times before capsaicin administration, 30, 45 and 60 minutes after capsaicin. Data represents mean maximum normalised increased responses compared to control readings when capsaicin was applied over the MMA (a) and into the FRF (b). Capsaicin administration in both sites resulted in a significant increase in brush and pinch stimulation compared to capsaicin vehicle, which was maximal thirty minutes after capsaicin application ($P < 0.05$; repeated measures ANOVA).

3.3.4 Immunohistochemistry

Following immunohistochemical processing of all tissue in these studies, *c-fos* expression was observed almost exclusively in lamina I / II, ipsilaterally to the stimulus.

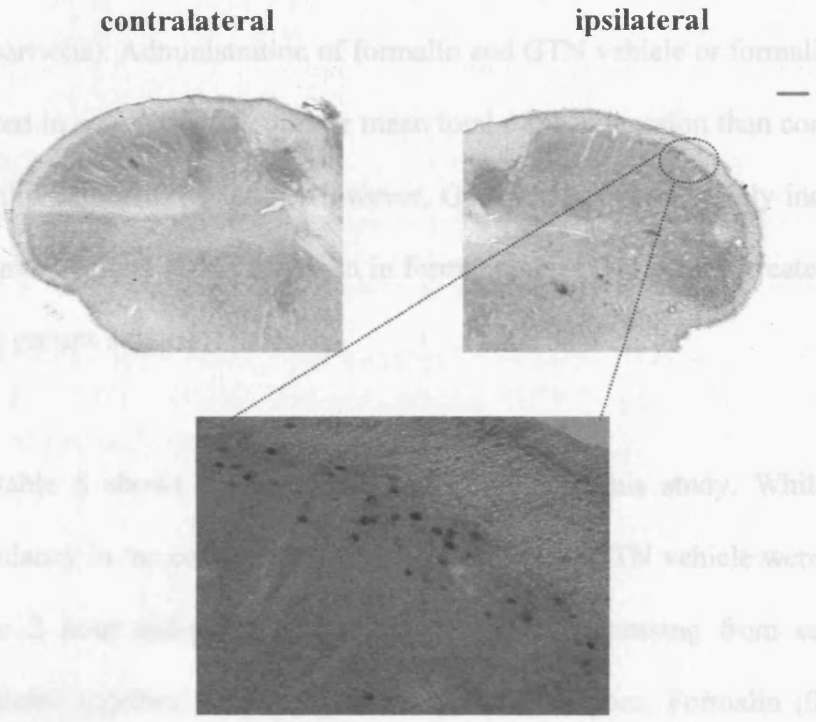


Figure 21 *c-fos* immunohistochemistry observed in the Vc

Picture shows c-fos expression in the superficial ipsilateral Vc, following application of capsaicin into the face. c-fos expression could not be observed in the contralateral Vc. Scale bar, 200μm

3.3.4.1 Formalin

Administration of formalin vehicle and GTN vehicle or formalin vehicle and GTN both generated an increase in mean total *c-fos* expression, however, this was not significantly different between the two treatment groups in tissue taken from animals euthanased, two or four hours after the end of the stabilisation period ($P > 0.05$, ANOVA, repeated measures followed by Bonferroni's *post hoc* test for multiple comparisons). Administration of formalin and GTN vehicle or formalin and GTN both resulted in a significantly greater mean total *c-fos* expression than combinations using formalin vehicle ($P < 0.05$). However, GTN did not significantly increase total *c-fos* immunoreactivity above that seen in formalin and GTN vehicle treated animals in any of the groups studied ($P > 0.05$).

The table 8 shows the total mean *fos* counts in this study. While there is some redundancy in the counts, i.e. formalin vehicle and GTN vehicle were looked at twice at the 2 hour end-point, this is the result of processing from each study being completed together. For example, in the first instance, Formalin (0.5%) / formalin vehicle and GTN / GTN vehicle infusion were examined and the tissue processed together. When no sensitising effect of GTN was seen, Formalin was then examined at 5%, however, because of variation in tissue processing, it was not considered acceptable to use data for formalin vehicle and GTN vehicle from the earlier study with formalin, 0.5%.

	2 hour time point		4 hour time point	
	0.5%	**5%**	**0.5%**	**5%**
Formalin vehicle & GTN vehicle	188 ± 54 (n = 5)	139 ± 36 (n = 5)	78 ± 11 (n = 5)	110 ± 48 (n = 5)
Formalin vehicle & GTN	212 ± 76 (n = 5)	187 ± 64 (n = 5)	129 ± 40 (n = 5)	137 ± 53 (n = 6)
Formalin & GTN vehicle	1011 ± 94 (n = 5)	1376 ± 151 (n = 5)	746 ± 110 (n = 5)	878 ± 142 (n = 5)
Formalin & GTN	1079 ± 202 (n = 5)	1402 ± 235 (n = 6)	587 ± 43 (n = 5)	767 ± 94 (n = 5)

Table 8 Total mean fos counts following treatment with formalin and GTN

Table shows total mean c-fos expression following treatment with combination of formalin (0.5% and 5%), formalin vehicle, GTN (5μgkg^{-1}min^{-1}) and GTN vehicle at both the two and four hour end point.

When data was considered as mean *c-fos* expression per section, formalin, 0.5% followed by GTN infusion (5μgkg^{-1}min^{-1}) resulted in a significant increase in *c-fos* positive nuclei in sections taken from two areas (0.97 and 0.85mm caudal to area postrema) compared to formalin, 0.5% and GTN vehicle infusion ($P < 0.05$, repeated measures ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons). This effect occurred in tissue taken from animals where euthanasia was carried out 4 hours after the stabilisation period and was not evident in the 2 hour study, or where formalin was used at a higher concentration of 5%.

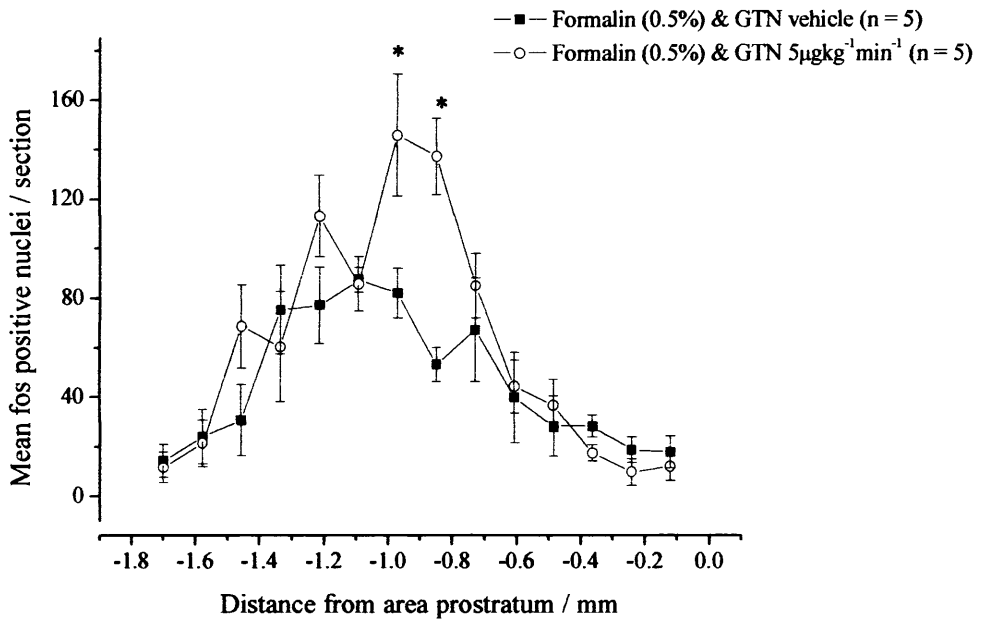


Figure 5 Facilitation by GTN of *c-fos* expression induced by formalin

c-fos expression was assessed as described in section 2.3. Data represents mean c-fos expression per section from animals treated with either formalin and GTN vehicle infusion or formalin and GTN infusion and perfused 4 hours after GTN infusion. The mean c-fos expression induced by formalin, was significantly facilitated by GTN infusion compared to vehicle infusion in two sections ($P < 0.05$ =, repeated measures ANOVA).*

3.3.4.2 Capsaicin

Mean total *c-fos* expression from all sections in each animal were compared in the different drug combination groups and between the different anaesthetic protocols (α-chloralose versus urethane). Mean total *c-fos* expression was not significantly different for tissue taken from treatment groups receiving capsaicin vehicle and GTN vehicle or capsaicin vehicle and GTN ($P > 0.05$). Treatment with capsaicin and GTN vehicle or capsaicin and GTN both resulted in a significantly increase in the mean total *c-fos* expression over either of the treatment groups with capsaicin vehicle ($P < 0.05$). However, GTN did not significantly increase total *c-fos* immunoreactivity above that seen in capsaicin and GTN vehicle treated animals ($P > 0.05$). There was also no effect of the different anaesthesia protocols on *c-fos* expression in any of the treatment groups ($P > 0.05$). Additionally, the absence of a stabilisation period after surgery did not appear to effect total *c-fos* expression.

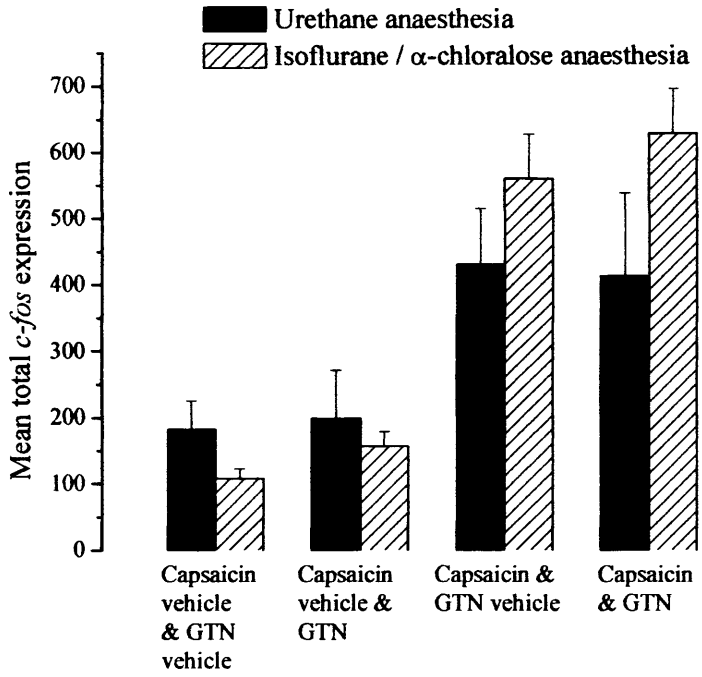


Figure 22 Total *c-fos* expression following GTN and capsaicin combinations

c-fos expression was assessed as described in section 2.3. Data represents total c-fos expression assessed at the 90-minute end point, following capsaicin vehicle and GTN vehicle infusion, capsaicin vehicle and GTN infusion, capsaicin and GTN vehicle infusion and capsaicin and GTN infusion. Statistical significance was assessed using a repeated measures ANOVA. There was no statistical significant difference in total c-fos expression seen in any treatment group in animals treated under either anaesthetic regime ($P > 0.05$). In both groups, capsaicin resulted in a significant increase in c-fos expression compared to animals receiving capsaicin vehicle ($P < 0.05$), however GTN failed to significantly increase total c-fos expression induced by capsaicin in any treatment group ($P > 0.05$).

While the total *c-fos* expression showed that GTN did not significantly increase *c-fos* immunoreactivity induced by capsaicin, there was some evidence for a facilitation by GTN when mean *c-fos* expression per tissue section were examined. This facilitation occurred in animals anaesthetised with isoflurane and α-chloralose. The facilitation was restricted to sections from two locations around area postrema where mean *c-fos* expression per tissue section following both GTN infusion and capsaicin injection were 93 ± 17 (n = 5) and 84 ± 16 (n = 5) compared to 56 ± 9 (n = 5) and 50 ± 14 (n = 5) following GTN vehicle infusion and capsaicin injection ($P < 0.05$).

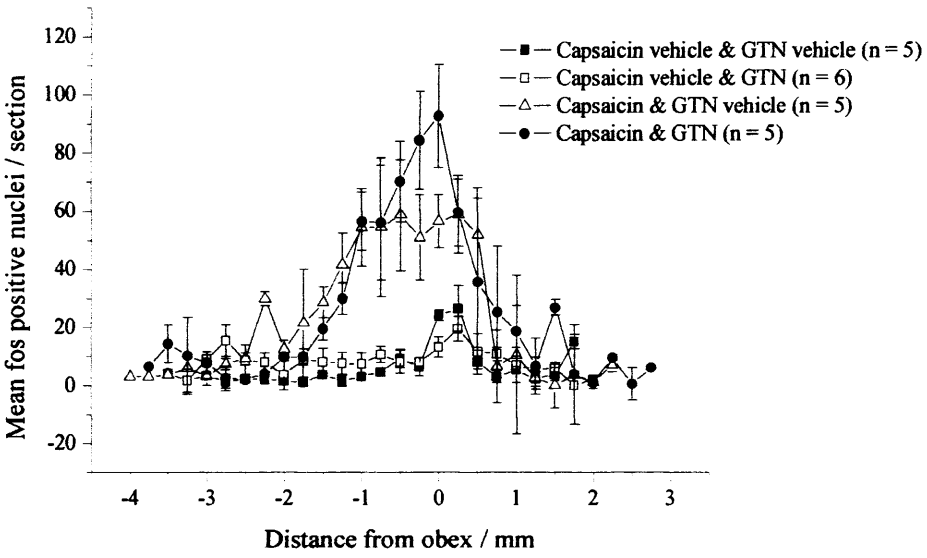


Figure 23 Facilitation by GTN of *c-fos* expression induced by capsaicin

c-fos expression was assessed as described in section 2.3. Data represents mean c-fos expression per section from animals anaesthetised with isoflurane and α-chloralose and treated with either i.) Capsaicin vehicle and GTN vehicle infusion, ii.) Capsaicin vehicle and GTN infusion, iii.) Capsaicin and GTN vehicle infusion and iv.) capsaicin and GTN infusion. The mean c-fos expression induced by capsaicin, was significantly facilitated by GTN infusion compared to vehicle infusion in sections taken from two areas ($P < 0.05$ =, repeated measures ANOVA).*

3.3.4.3 PGE$_2$ and NGF

The mean total *c-fos* expression following administration of NGF with PGE_2 and NGF with PGE_2 vehicle was not altered by either GTN infusion ($5\mu gkg^{-1}min^{-1}$) or GTN vehicle infusion (each treatment group, n = 3). However, following administration of PGE_2 and NGF vehicle, GTN infusion result in a increase in mean total *c-fos* expression compared to *c-fos* expression following administration of PGE_2 and NGF and GTN vehicle (33 ± 7 and 16 ± 3, respectively).

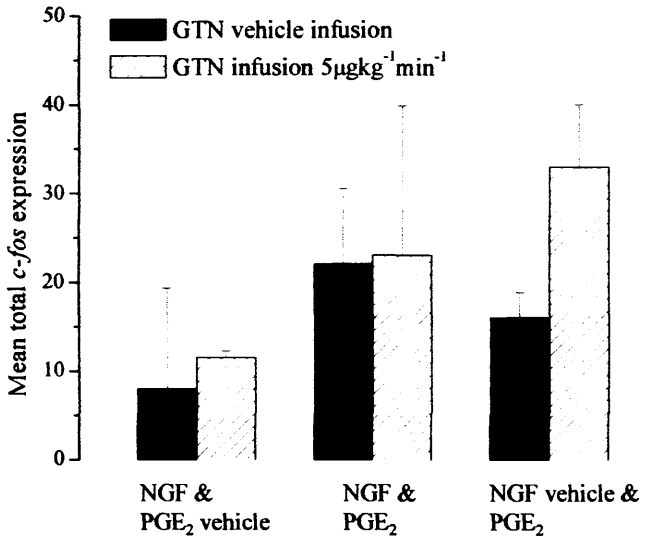


Figure 24 *c-fos* expression following administration of PGE_2, GTN and NGF

c-fos expression was assessed as described in section 2.3. Data represents total c-fos expression assessed at the 2-hour end point following application of either NGF and PGE_2 vehicle or NGF and PGE_2 or NGF vehicle and PGE_2 into the face.

3.4 Discussion

3.4.1 Intra-arterial GTN infusions

These result in section 3.3.2.1.demonstrate that GTN infusion is able to alter the activity of second order neurons receiving input from the ophthalmic division of the trigeminal nerve, both during and following the infusion. The observation of both inhibitory and excitatory effects on neuronal activity is consistent with previous studies that have described a similar effect following intravenous infusions of GTN; an increase in neuronal activity in 4/5 cells tested and an inhibition of neuronal response in 1/5 cells (Jones *et al.*, 2001). This variability may be the result of recording from functionally distinct second order cells within the Vc and indeed evidence exists for a differential action of nitric oxide on the firing of neurons elsewhere in the spinal cord (Pehl & Schmid, 1997). Although the recording position of each neuron was not examined immunohistochemically, the depth of recording from the pial surface gives an indication of the lamina position, with the highest concentration of lamina I and II neurons found between 0 and 250μm and lamina V-VI neurons between 500 and 1000μm below the pial surface (Urch & Dickenson, 2003). Indeed, cells in this study appeared to fall into superficially located cells (65 to 295μm) and deeper located cells (780 to 1025μm). However, there was no correlation between cells recorded in superficial and deep layers with the response to GTN. The variability in the response to GTN may also be related to the differential modulation of Vc neurons by descending pathways and studies using GTN infusion in decerebrate animals, where the influence of higher order structures is removed, would likely provide an insight

into this.

Topical GTN administration into the peripheral receptive field around the MMA (section 3.3.2.2) was almost identical to the effect of intra-arterial infusions; hence, a peripheral effect may be responsible for the observed effects on neuronal activity, although, given the highly lipophilic nature of GTN, a central effect cannot be ruled out. GTN infusion at a rate of $5\mu gkg^{-1}min^{-1}$ did not increase MMA diameter as assessed by intravital microscopy (section 3.3.1), but nonetheless altered MMA-evoked firing in Vc neurons (section 3.3.2.1), hence, increased Vc neuronal activity is not caused simply by dilatation of the MMA with an associated increase in primary afferent neurone firing. This is in agreement with recent clinical findings showing that GTN does not alter middle cerebral artery diameter but robustly generates migraine in 2 / 3 of migraineurs studied (Goadsby *et al.,* 2004). Therefore, it seems likely that in the time period examined, GTN is exerting a direct effect on primary afferent neurons.

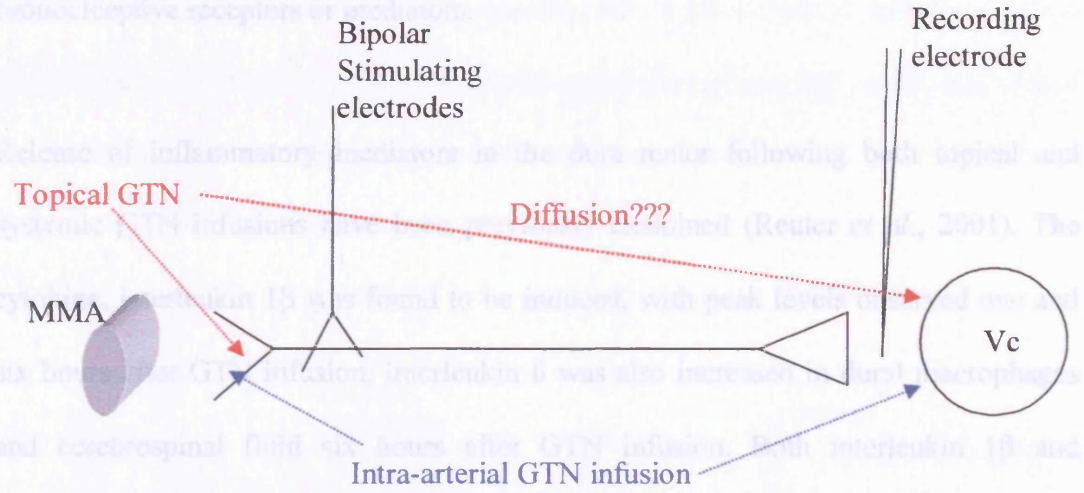


Figure 25 Site of action of GTN

The above schematic shows the recording set up for electrophysiological studies described. Both Topical and intra-arterial infusion of GTN resulted in a significant alteration of MMA-evoked Vc firing, however, intra-arterial infusion of GTN (5μgkg^{-1}min^{-1}) did not result in dilatation of the MMA, indicating that GTN acts directly on the peripheral terminal of the primary afferent neuron, although due o the lipophilicty of GTN, tissue diffusion and a central action cannot be ruled out.

Administration of CGRP in these studies resulted in vasodilatation of the MMA in agreement with previous studies (section 3.3.1.1; Cumberbatch *et al.*, 1999). Interestingly, following this dilatation, a second GTN infusion resulted in vasodilatation of the MMA during infusion at 5μg kg^{-1}min^{-1}, which in the first infusion had no effect on MMA diameter. Hence, it is likely that either the first series of GTN infusions or CGRP generated a higher sensitivity to the effects of GTN. This increased sensitivity may underlie the delayed headache seen clinically following GTN infusion

in migraineurs, for example, GTN facilitated release of CGRP or other inflammatory mediators may ultimately lead to an alteration in gene expression and up-regulation of pronociceptive receptors or mediators.

Release of inflammatory mediators in the dura mater following both topical and systemic GTN infusions have been previously examined (Reuter *et al.*, 2001). The cytokine, interleukin 1β was found to be induced, with peak levels observed one and six hours after GTN infusion, interleukin 6 was also increased in dural macrophages and cerebrospinal fluid six hours after GTN infusion. Both interleukin 1β and interleukin 6 are known to be involved in the development of oedema and Aδ sensitisation (Macmicking *et al.*, 1997). Dose dependant increases in Type II nitric oxide synthase (or inducible nitric oxide synthase) mRNA was also detected in the dura mater at two hours with corresponding increases in the protein at four, six and ten hours after GTN infusion. However, whether interleukin or nitric oxidase synthase up-regulation occurs differentially in migraine sufferers and healthy volunteers remains to be explored.

3.4.2 The effect of GTN in combination with inflammatory mediators

The application of capsaicin to the dural receptive field resulted in a significant facilitation of evoked Vc responses (section 3.3.3.2.1). Significant enhancement of FRF responses was also seen, indicating the development of central sensitisation (section 3.3.3.2.4.). However, when capsaicin was administered into an area outside the FRF facilitation of electrically evoked MMA-responses was much less pronounced (section 3.3.3.2.3.) although significant *c-fos* expression could be observed following

the same capsaicin stimulus (section 3.3.4.2.). This suggests that while capsaicin administration into the facial skin can generate a robust central neuronal activation, the pool of neurons activated may not coincide with those activated following MMA stimulation. Alternatively, the differential response following dural and facial capsaicin application may represent different sensitivities of the primary afferent neurons to capsaicin stimulation.

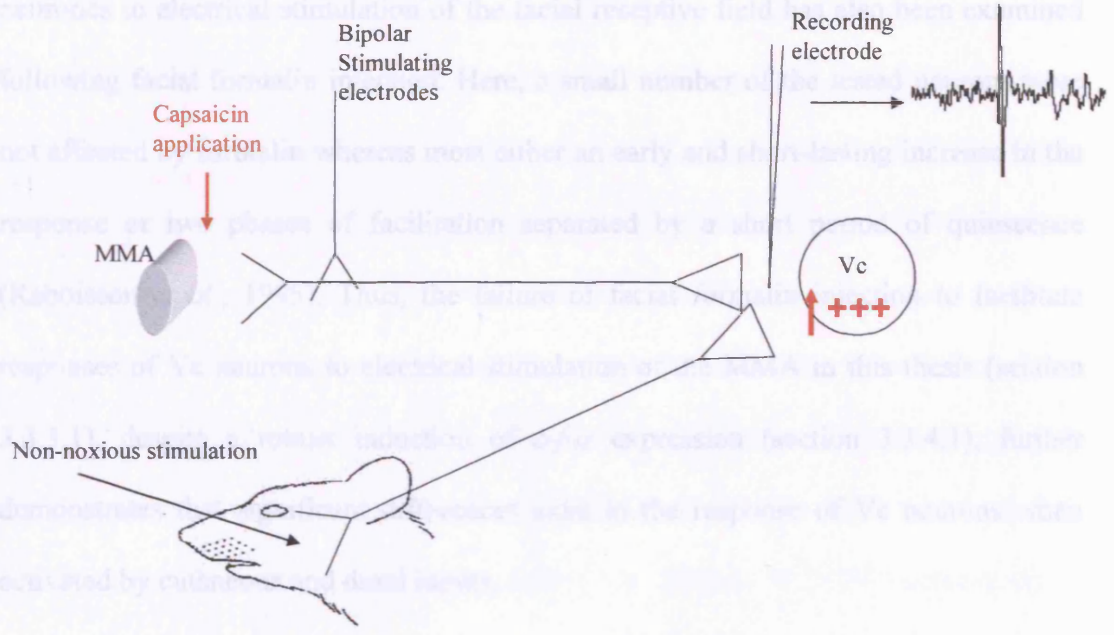


Figure 26 Topical capsaicin application results in central sensitisation

Capsaicin application over the MMA resulted in a significantly facilitated response both to MMA-evoked firing and the response to facial stimulation indicating the development of central sensitisation.

Within the rat, trigeminal system formalin has been examined as a sensitizer of cutaneous afferents (Clavelou *et al.*, 1989) and of deeper tissue afferents in the temporomandibular joint (Roveroni *et al.*, 2001) with enhanced nociceptive behaviour

observed in both models. Furthermore, following sub-cutaneous facial formalin injection, considerable *c-fos* expression can be observed in laminae I, II and V of the VBNC as well as in the adjacent ventrolateral reticular formation (Wang *et al.*, 1994). Interestingly, cells exhibiting *c-fos* expression following facial injection of formalin, were co-localised with the glutamate NMDA receptor (94% of cells showing *c-fos* immunoreactivity) and with NO synthase (14% of cells showing *c-fos* immunoreactivity) (Leong *et al.*, 2000). The electrophysiological response of Vc neurones to electrical stimulation of the facial receptive field has also been examined following facial formalin injection. Here, a small number of the tested neurons were not affected by formalin whereas most either an early and short-lasting increase in the response or two phases of facilitation separated by a short period of quiescence (Raboisson *et al.*, 1995). Thus, the failure of facial formalin injection to facilitate responses of Vc neurons to electrical stimulation of the MMA in this thesis (section 3.3.3.1), despite a robust induction of *c-fos* expression (section 3.3.4.1), further demonstrates that significant differences exist in the response of Vc neurons when activated by cutaneous and dural inputs.

In the study designed to replicate the facilitation by GTN of *c-fos* expression induced by capsaicin, described by Jones *et al.* (2001), the authors were contacted to ensure identical conditions (i.e. rat strain and supplier, anaesthetic doses). However, GTN infusion failed to increase total *c-fos* expression induced by capsaicin in any experiments of this thesis (section 3.3.4.2.), moreover GTN had no effect on capsaicin induced sensitisation of Vc electrophysiological response (section 3.3.3.2.2). This lack of facilitation of total *c-fos* expression by GTN was also evident in studies using formalin to stimulate *c-fos* expression (section 3.3.4.1.). However, facilitation by GTN

of *c-fos* expression induced by capsaicin and formalin was observed in sections from two areas, which corresponded to the Vc. The facilitation was more evident in the experiments using formalin rather than capsaicin as a mediator of sensitisation. However, this may be related to the end-points of the experiment, as tissue was taken from animals four hours after GTN infusion in the formalin study but in order to allow comparison of data in the capsaicin study with a previously published study (Jones *et al.*, 2001), the end-point was ninety minutes.

The facilitation of *c-fos* expression following GTN infusion, that is highly specific to the Vc and maximal four hours after GTN infusion, supports the hypothesis that in migraine subjects, GTN is specifically able to act on a subset of neurons that are more sensitised than in controls to generate a delayed headache. The origin of the central sensitisation is still a subject of speculation; however, following completion of this thesis, an up-regulation of the 5-HT_{2A} receptor in migraineurs has been proposed to underlie the delayed headache response to GTN (Srikiatkhachorn *et al.*, 2002). Although this hypothesis is still speculative, the efficacy if 5-HT_2 antagonists in migraine prophylaxis (see section 1.8.4.3.) make this an attractive prospect.

Sensitisation elicited by PGE_2 application into the division of V_2 was also facilitated following GTN infusion. However, the numbers in this study were small and further experimental data is needed to support these results. As such, study 2 in this thesis has focused on sensitisation generated by prostanoids in the trigeminal system. Evidence for a role of prostaglandins in mediating the biological effect of GTN were implicated in a recent study (Tassorelli *et al.*, 1997). Here, indomethacin, which inhibits the cyclo-oxygenase (COX) enzyme, responsible for the production of prostaglandins,

resulted in a significant reduction in *c-fos* expression in the Vsp and several supraspinal nuclei compared to when GTN was administered alone. Other evidence has also emerged in the last few years that further implicate prostaglandins in the pathology of migraine and this is discussed in section 4.1.

In summary, the differential response following GTN infusion in healthy volunteers and migraineurs can not yet be adequately explained by available animal models. It is possible that migraineurs have a greater susceptibility to cytokines and other inflammatory mediators released in the dura mater following GTN infusion. However, it is also possible that peripheral activation occurs in a similar manner in both healthy volunteers and migraineurs and the difference lies in the response of either second or third order neurons, which may be modulated by receptor expression such as the 5-HT_{2A} receptor. It is also possible that the effects of GTN infusion trigger a migrainous cascade within the central nervous system completely unrelated to any putative inflammatory mechanism.

Treatment	Summary
Intra-arterial / topical GTN infusion - Intravital microscopy	Vasodilatation maximally following CGRP and topical GTN application. Possibly greater vasodilatation after sensitization
Intra-arterial GTN infusion -Vc electrophysiology	Inhibitor and excitatory response seen
Capsaicin over the MMA and intravenous GTN infusion - Vc electrophysiology	Sensitization of both electrical evoked and mechanically evoked response. Not facilitated by GTN
Capsaicin into the FRF and intravenous GTN infusion - Vc electrophysiology	Sensitization mechanically evoked and electrically evoked response
Formalin into the FRF and intravenous GTN infusion - Vc electrophysiology	No sensitization seen
Formalin into the FRF and intravenous GTN infusion - *c-fos* immunohistochemistry	Robust *c-fos* expression following formalin. Some evidence for facilitation by GTN in specific discrete area of the Vc
Capsaicin into the FRF and intravenous GTN infusion - *c-fos* immunohistochemistry	Robust *c-fos* expression following capsaicin. Some evidence for facilitation by GTN in specific discrete area of the Vc
PGE_2 and NGF into the FRF and intravenous GTN infusion - *c-fos* immunohistochemistry	Numbers small but *c-fos* expression induced by PGE_2 may be facilitated by GTN

Table 9 Results summary of study 1

4 Study 2 The Role of Prostanoids in sensitisation of the trigeminovascular system

4.1 Introduction

The role of prostaglandins (PGs) in causing pain has been the subject of much research since the proposal in the early 1970's that aspirin-like drugs exert their effects by interfering with their production (Vane, 1971; Ferreira, 1972). PGs were first discovered in the 1930's by a group of medical students, who identified them as an active substance present in semen that was able to cause contraction of uterine smooth muscle. During the 1960's, the crystalline form of PGE_2 and $PGF_{2\alpha}$ were identified (Bergstorm & Sjovall, 1960), and some years later it was discovered that PGs were part of a large class of compounds, now known as eicosanoids. The eicosanoids, as well as PGs, encompass other classes of compounds such as thromboxanes, leukotrienes and lipoxins, all of which are derived from the unsaturated fatty acid, arachidonic acid (AA). AA itself is liberated from cell membranes by the action of various phospholipases, following cellular activation by various physiological and pathophysiological factors such as inflammation, hormonal influences and increased intracellular calcium.

Following the liberation of AA from the cell membrane, intermediate products PGG_2 and PGH_2 are produced by the action of an enzyme, variously known as prostaglandin H synthase or prostaglandin endoperoxidase synthase or more commonly as cyclo-oxygenase (COX). Further enzymatic conversion by various prostaglandin synthases, isomerase and thrombaxane synthases, gives rise to the functionally active eicosanoids, which are released from the cell following synthesis.

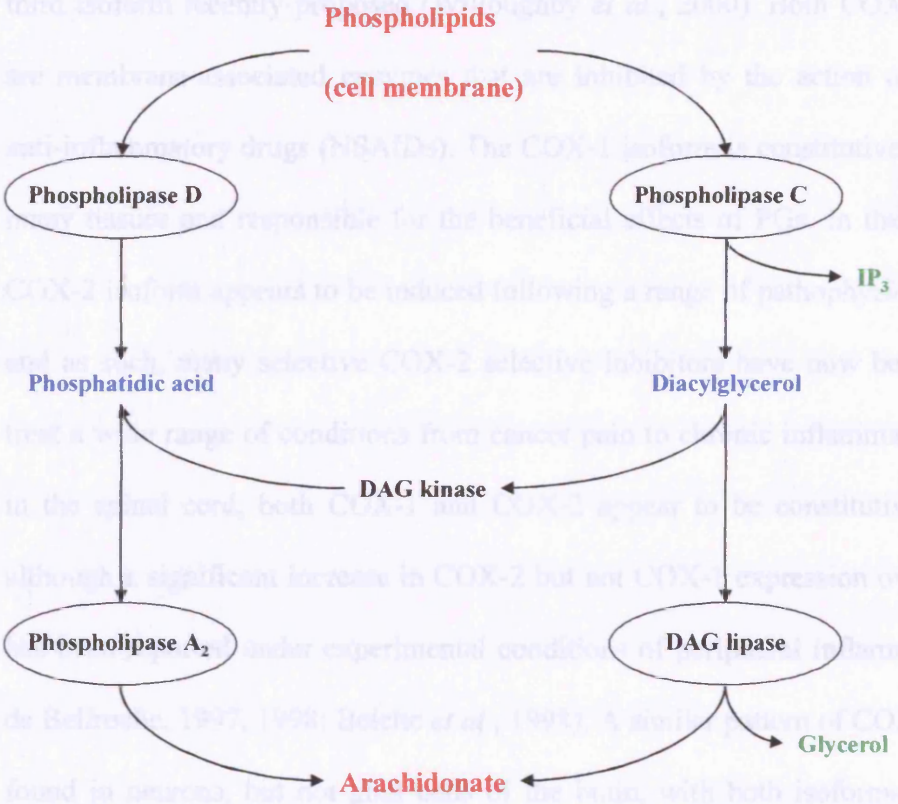


Figure 27 Liberation of AA from cell membranes by the action of phospholipases

The initial and rate limiting step in eicosanoid synthesis is the release of AA from cell membranes. Arachidonic acid (or arachidonate) is liberated from cell membranes in either a one-step process, involving phospholipase A_2 or a two step process involving either phospholipase C and then DAG lipase or phospholipase D and then phospholipase A_2.

At least two types of the COX enzyme are known to exist (COX-1 and COX-2), with a third isoform recently proposed (Willoughby *et al.*, 2000). Both COX-1 and COX-2 are membrane-associated enzymes that are inhibited by the action of non-steroidal anti-inflammatory drugs (NSAIDs). The COX-1 isoform is constitutively expressed in many tissues and responsible for the beneficial effects of PGs. In the periphery, the COX-2 isoform appears to be induced following a range of pathophysiological stimuli and as such, many selective COX-2 selective inhibitors have now been designed to treat a wide range of conditions from cancer pain to chronic inflammation. However, in the spinal cord, both COX-1 and COX-2 appear to be constitutively expressed, although a significant increase in COX-2 but not COX-1 expression over basal levels has been reported under experimental conditions of peripheral inflammation (Hay & de Bellroche, 1997, 1998; Beiche *et al.*, 1998). A similar pattern of COX expression is found in neurons, but not glial cells of the brain, with both isoforms present under basal conditions and an up-regulation of COX-2 in response to stress (Yamagata *et al.*, 1993).

The functional metabolic PG products of arachidonic acid have been classified as types D, E, F and I and once released into the intracellular space, by an as yet, undefined mechanism, exert their effects by acting on specific receptors in the plasma membrane. The receptors for these functional PGs are designated by a "P" and preceded by their main agonist type of PG, i.e. PGD_2, PGE_2, PGF_2 and PGI_2 act on DP, EP, FP and IP receptors respectively. All of these receptors are G-protein coupled receptors and exert their effects by activating intracellular signalling cascades depending on their G-protein coupling. Studies in this thesis have focused on PGE_2 only, hence, the following review will concentrate on the EP receptors, although it

should be remembered that there is considerable evidence to suggest cross-reactivity of PGE_2 with other PG receptors.

There are four main sub-types of the EP receptor, namely EP_1, EP_2 EP_3 and EP_4 (Kennedy *et al.*, 1982), each having distinct signal transduction pathways. These signal transduction pathways have been investigated following agonist induced changes in the levels of second messengers (for review see Narumiya *et al.*, 2001). EP_1 activation appears to result in an increase in Ca^{2+} mobilisation with subsequent PKC activation, EP_2 and EP_4 are coupled through Gs to increase cAMP and EP_3 has several splice variants as a result of post-translational modification that can either increase intracellular Ca^{2+}, or inhibit or stimulate cAMP.

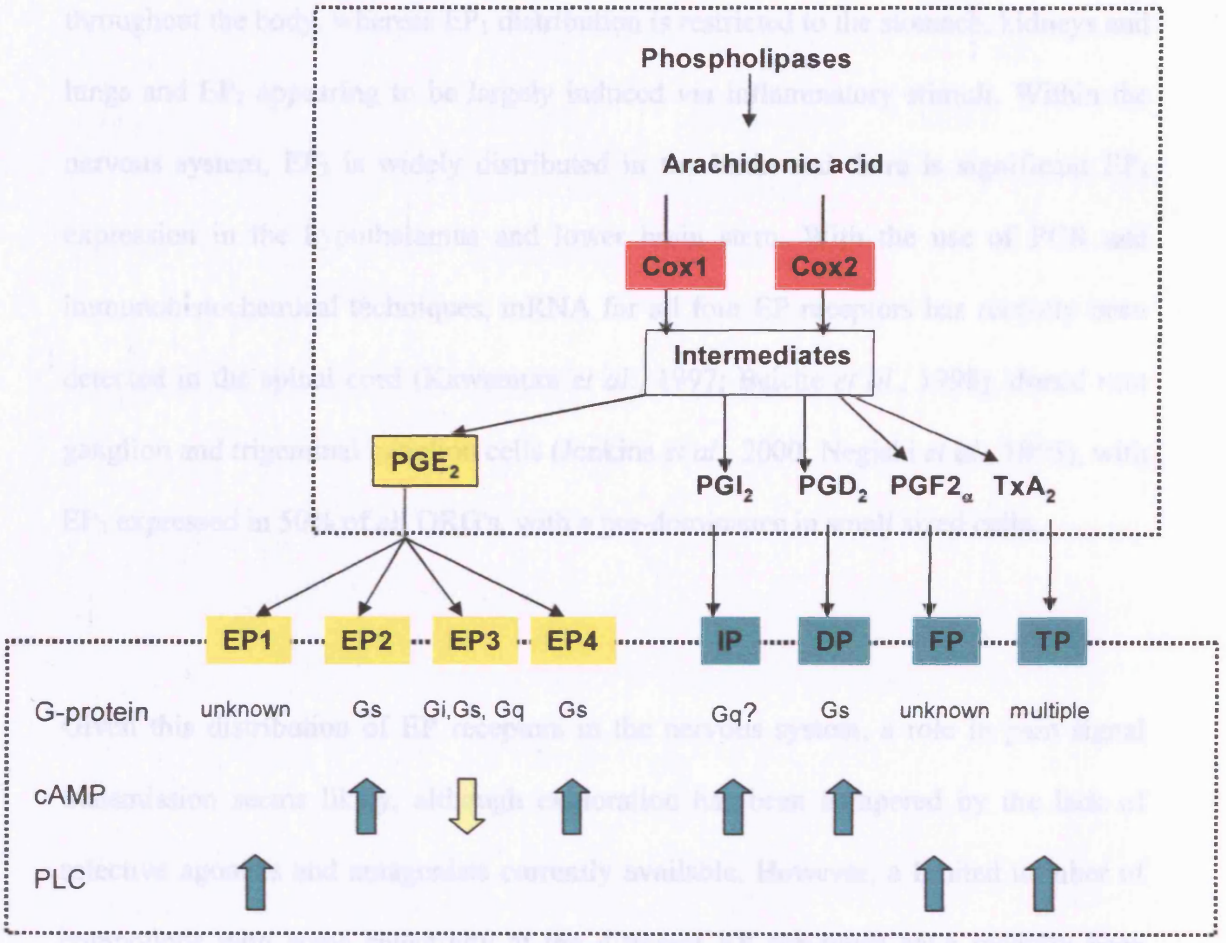


Figure 28 Prostaglandin synthesis

Following release of AA into the cytosol, cyclo-oxygenase enzymes act in a two step process to produce PGG_2 which is further converted to PGH_2. The fate of these intermediate products then varies between cell types, with various prostaglandin synthases producing the various functional products.

Of the EP receptors, EP_3 and EP_4 receptors are widely distributed in most tissues throughout the body, whereas EP_1 distribution is restricted to the stomach, kidneys and lungs and EP_2 appearing to be largely induced via inflammatory stimuli. Within the nervous system, EP_3 is widely distributed in the brain and there is significant EP_4 expression in the hypothalamus and lower brain stem. With the use of PCR and immunohistochemical techniques, mRNA for all four EP receptors has recently been detected in the spinal cord (Kawamura *et al.*, 1997; Beiche *et al.*, 1998), dorsal root ganglion and trigeminal ganglion cells (Jenkins *et al.*, 2000; Negishi *et al.*, 1995), with EP_3 expressed in 50% of all DRG's, with a pre-dominance in small sized cells.

Given this distribution of EP receptors in the nervous system, a role in pain signal transmission seems likely, although exploration has been hampered by the lack of selective agonists and antagonists currently available. However, a limited number of compounds with some selectivity at the different EP sub-types have recently been made accessible and are summarised in the table below.

	EP_1	EP_2	EP_3	EP_4
Agonists	-17-phenyl-trinor-PGE_2 -iloprost -sulprostone	-Butaprost -AH13205 -Misoprostol	-Sulprostone -SC-46275 -ONO-NT-012 -SC 46275	-NONE
Antagonists	-ZM325802 -SC51322 -SC19220 -ZD6416 -AH6809 -ONO-NT-012	-AH6809	-NONE	-GW627368 -AH22921 -AH23848

Table 10 Ligand availability for the EP receptors

Summary of available ligands acting on EP receptors; Iloprost is a partial agonist at EP_1 receptors but also a potent full agonist at IP receptors; Misoprostol also has an effect at EP_3 receptors; AH-22921 and AH-23848 are also antagonists at TP receptors.

Prostanoids contribute to the development of pain by acting both peripherally and centrally. Peripherally, they play a major role in generating peripheral sensitization by increasing the sensitivity of the peripheral terminals of high-threshold pain fibres. Centrally administered PGs produce marked alterations in pain behaviour, including exaggerated responses to noxious stimuli as well as pain responses to normally innocuous stimuli.

PGs, mainly PGE_2 and PGI_2, are rapidly produced following tissue injury or inflammation at the site of injury in response to noxious mechanical, thermal, and chemical stimuli. Bradykinin is one of the most potent algogans and is considered to mediate hyperalgesia during inflammatory processes and partly depend on a sensitization action of PGE_2. PGs can be also released within the spinal cord by noxious, but not innocuous, stimuli applied to peripheral tissues. Enhanced spinal release of PGE_2 and induction of COX-2 in the spinal cord are major consequences of peripheral inflammation, and these two events may be linked when inflammation is prolonged. (for review see Ito et al., 2001). The action of PGs in generating peripheral inflammation, oedema and localized swelling following tissue injury is well known, however, the source and mechanism of action of PGs in the inflammation process is not yet well characterized. A recent study showed that both macrophages and mast cells are likely to be a source, although other sites of production such as sensory neurons and vascular endothelium are also likely to be involved in their release (Ma & Eisenach, 2002).

The mechanism by which PGs generate peripheral sensitisation was initially thought to result from induction of a heightened responsiveness of sensory neurons to the

effects of other mediators, although data now indicate that PGE_2 is able to cause a direct activation of sensory neurons (Baba *et al.*, 2001). The cellular mechanism by which PGs exert their effects on peripheral neurons is thought to involve activation of several different ion channels with resultant activation of the cAMP second messenger system and neuropeptide release, which has been confirmed in *in vitro* experiments by the use of PKA inhibitors (Aley & Levine, 1999). PGE_2 has also been shown to alter the activation potential of tetrodotoxin-resistant sodium currents (TTX-R I_{Na}) to more negative potentials (England *et al.*, 1996), facilitate the capsaicin-induced current (Lopshire & Nicol, 1998), increase the activation of the hyperpolarization-activated cation current (I_h; Ingram & Williams, 1996) and attenuate calcium-activated potassium currents (Gold *et al.*, 1996). The ability of PGE_2 to modulate the activity of the TTX-R I_{Na} through a PKA-related mechanism has been further explored by studies using knockout mice deficient in the SNS channel (sensory neuron-specific tetrodotoxin (TTX)-resistant voltage-gated sodium channel), responsible for the TTX-R I_{Na} and in mice deficient for RI, a regulatory subunit of the PKA enzyme. In these mutant mice, a defective nociceptive response following intraplantar PGE_2 was seen, compared to wild type mice, indicating PKA phosphorylation of sensory neurone membrane channels is likely to be involved in the sensitising effects of PGE_2 (Malmberg *et al.*, 1997). Further determination of the role of PGs in peripheral sensitisation, may be aided by the use of specific knockout mice deficient in the EP_1, EP_2 (Kennedy *et al.*, 1999), EP_3 (Ushikubi *et al.*, 1998) and EP_4 (Sakuma *et al.*, 1994) receptors, however at present, the peripheral site of PG action in generating pain remains largely undiscovered.

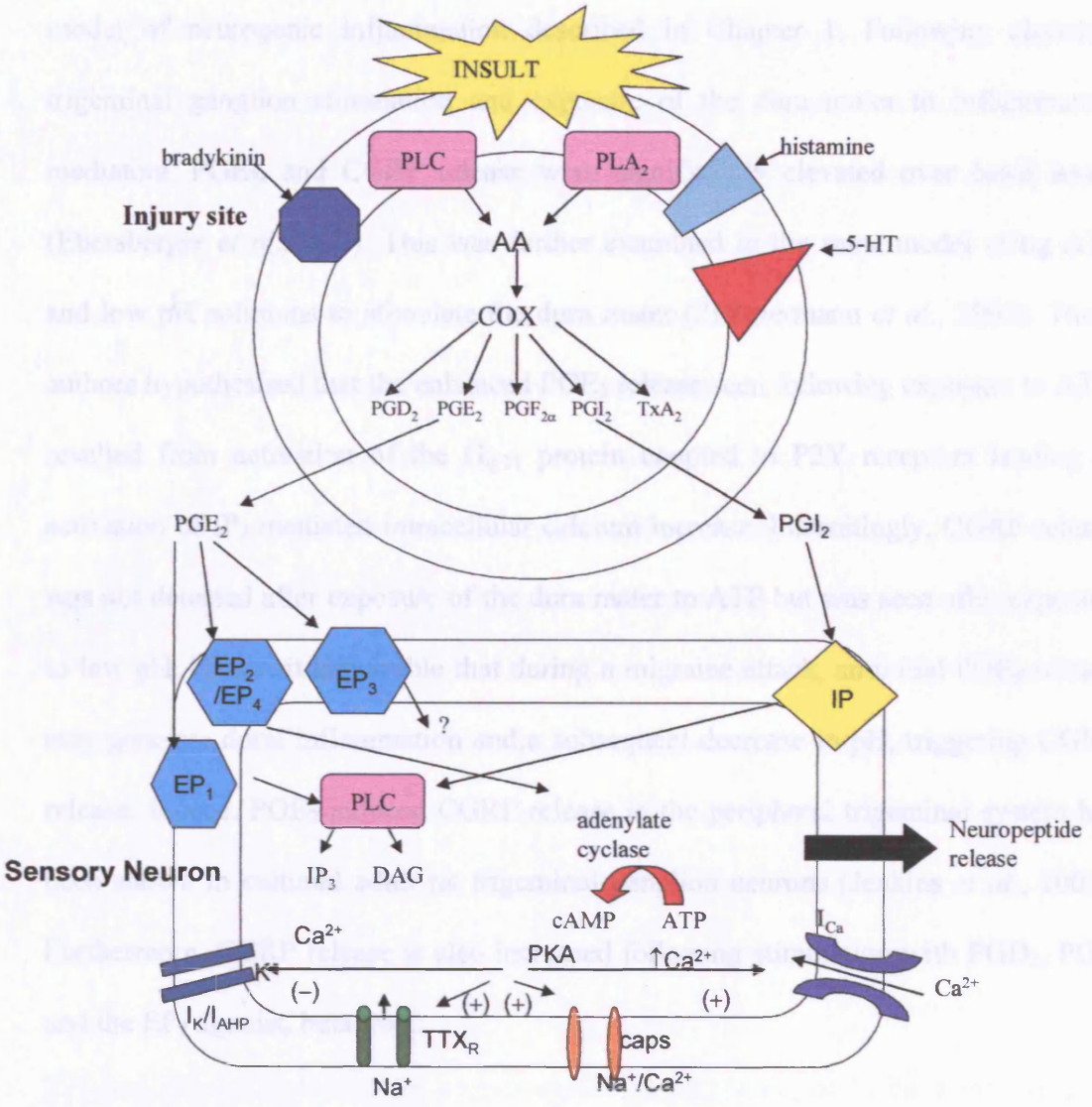


Figure 29 Peripheral sensitisation following PG release

The mechanism of prostaglandins induced sensitisation of peripheral neurons is not fully understood. However preliminary investigation has shown that following cellular insult, prostaglandins are released into the extracellular space and act on cell surface receptors to cause a variety of intracellular effects in surrounding sensory neurons such as activation of protein kinases and subsequent modulation of ion channels.

The involvement of PGE_2 in dural sensitisation has been demonstrated in the *in vitro* model of neurogenic inflammation described in Chapter 1. Following electrical trigeminal ganglion stimulation and exposure of the dura mater to inflammatory mediators, PGE_2, and CGRP release were significantly elevated over basal levels (Ebersberger *et al.*,1999). This was further examined in the same model using ATP and low pH solutions to stimulate the dura mater (Zimmermann *et al.*, 2002). These authors hypothesised that the enhanced PGE_2 release seen following exposure to ATP, resulted from activation of the $G_{q/11}$ protein coupled to P2Y receptors leading to activation of IP_3 mediated intracellular calcium increase. Interestingly, CGRP release was not detected after exposure of the dura mater to ATP but was seen after exposure to low pH. Hence, it is possible that during a migraine attack, an initial PGE_2 release may generate dural inflammation and a subsequent decrease in pH, triggering CGRP release. Indeed, PGE_2-induced CGRP release in the peripheral trigeminal system has been shown in cultured adult rat trigeminal ganglion neurons (Jenkins *et al.*, 2001). Furthermore, CGRP release is also increased following stimulation with PGD_2, PGI_2 and the EP_2 agonist, butaprost.

As previously discussed, PGE_2 also forms part of the "soup" of inflammatory mediators, that when applied to an open cranial window generates an increase in the response of second order neurons within the Vc (Burstein *et al.*, 1998). Given the evidence now available from *in vitro* studies to support a role of PGE_2 in initiating dural inflammation, this study has focused on the specific ability of PGE_2 to sensitise the trigeminal system, evidenced by an increased activity of second-order neurons in the Vc.

Additionally the effect of, the efficacious migraine treatment, naratriptan (see section 1.8.3.2.) has been examined. Naratriptan (N-methyl-3-(1-methyl-4-piperidinyl)-1H-indole-5-ethane-sulphonamide), a 5HT1B/1D receptor agonist has no effect in a variety of antinociceptive tests but selectively inhibits neurogenic plasma protein extravasation in the dura in anaesthetized rats (ID50 = 4.1 micrograms kg-1) (Connor *et al.*, 1997). Additionally, naratriptan has previously been shown to inhibit Vc response to dural stimulation in the rat and cat (Cumberbatch *et al.*, 1998; Goadsby & Knight, 1997), moreover this effect appears specific for the Vc as dorsal horn responses to noxious stimulation of the hindpaw were unaffected by naratriptan (Cumberbatch *et al.*, 1998). The purpose of using naratriptan following the induction of sensitisation with PGE_2 in this study was to look for any differential effect compared to the efficacy of naratriptan in the un-sensitised state, thereby examining the possibility that the induction of sensitisation in migraineurs may be responsible for the proportion of migraineurs that are unresponsive to triptan therapy.

EP_1 and EP_4 antagonists were also examined in this model of PGE_2 sensitisation to determine whether PGE_2 was acting through either of these receptors. Due to the lack of selective EP_2 antagonists, the selective EP_2 agonist, butaprost was also examined for it's ability to generate sensitisation in a similar manner to PGE_2. The possibility that PGE_2 was acting through the EP_3 receptor was not examined due to the lack of availability of selective compounds.

4.2 Methods

Electrophysiological recording of second order neurons in the Vc responding to electrical stimulation of the MMA with Aδ-fibre latency were carried out as described in section 2.1.

4.2.1 Receptive field characterisation

Following identification of a Vc neuron that responded to electrical stimulation of the MMA, mechanical stimulation of the face was used to test for a convergent cutaneous input and only cells activated both by dural and facial stimulation were studied. The area of the face that was most sensitive to afferent stimulation was then marked on the face by marker pen (area typically 1 cm) and the response to a light, non-noxious 10-second brush and a noxious 5-second pinch stimulation of this area, assessed three times. The preparation was left for 10 minutes to minimize sensitisation from FRF stimulation, before trains of electrical stimulation (50 repetitions at 1Hz, 5 minutes apart) were applied to the dura mater overlying the MMA. No attempt was made to follow changes in FRF size during the course of an experiment, as in characterisation studies of Vc electrophysiology, considerable variation was found in FRF size after noxious stimulation such as pinch responses, which is in agreement with findings made by Dubuisson *et al.*, (1979). FRF's responses to brush and pinch stimuli were re-tested 30 minutes after PGE_2 stimulation and then at 15-minute intervals for the remainder of the experiment.

4.2.2 Evaluation of the sensitising effects of PGE_2

PGE_2 or PGE_2 vehicle was applied either topically over the MMA or subcutaneously injected into the FRF or given as an intra-arterial infusion following establishment of baseline recording to trains of electrical stimulation where a variation of less than 10% was seen). For topical application, a gauze swab (approx. 5mm x 5mm), containing 100µl of PGE2, 1mM, or PGE_2 vehicle (1% ethanol, 99% saline) was applied on the thinned cranial window below the bipolar electrodes for 5 minutes. A higher concentration of PGE_2 was used for topical application experiments compared to subcutaneous injection into the FRF or for the intra-arterial infusions due to the unreliability of compound penetration through the cranial window. During PGE_2 application, electrical stimulation was turned off to avoid problems with spread of the electrical stimulus to adjacent muscles. The gauze was then removed, the skull rinsed with saline, dried and re-covered with mineral oil. A caveat of this study was the use of ethanol as part of the PGE_2 vehicle, as alcohol is known to be a trigger for migraine (Martelletti *et al.*, 1991). However, the concentration of ethanol used was low and in control experiments, vehicle had no significant effect on Vc firing, although the possibility that a facilitation of the PGE_2 effect was related to the ethanol vehicle cannot be excluded.

For FRF application, PGE_2 (0.1mM in 50 µl) or PGE_2 vehicle (1% ethanol, 99%, saline in 50 µl) was administered subcutaneously into the area of the FRF that was most sensitive to afferent stimulation. For intra-arterial infusions of PGE_2, a second cannula was placed in the same carotid artery, cannulated for blood pressure monitoring, towards the cranial circulation. A constant pressure was applied by a slow

saline infusion (0.4ml/hr) to prevent back-flow from the circulation. PGE_2 (0.1mM) or PGE_2 vehicle was then administered over 5 minutes in 0.1ml. Following PGE_2 or PGE_2 vehicle administration, electrical stimulation trains were continued for the remainder of the experiment.

4.2.3 Naratriptan inhibition of PGE_2 sensitised responses

In separate experiments, the inhibitory effect of Naratriptan ($3mgkg^{-1}$ in 0.2ml or Naratriptan vehicle in 0.2ml, administrated as a bolus intravenous dose), was also tested. Experiments were carried out as described for topical application of PGE_2 and naratriptan administered immediately after application of PGE_2 or PGE_2 vehicle.

4.2.4 Prostanoid receptor subtypes involved in PGE_2- sensitisation

4.2.4.1 EP_2 agonist

Due to lack of selective EP_2 antagonists available, the EP_2 agonist, butaprost was assessed for its ability to generate sensitisation in comparison to PGE_2. Both topical administration (100µl of 5nM solution) and intra-arterial infusion (0.1 ml of 5nM solution over 5 minutes) were tested using the protocol described for PGE_2 above. The vehicle used to dissolve butaprost was the same as for PGE_2 (1% ethanol and 99% saline for final dilutions).

4.2.4.2 EP_1 antagonist

ZM325802 (EP_1 antagonist) or vehicle (1% dimethyl sulfoxide (DMSO), 66% PEG_{200} and 33% distilled water) was applied topically (5mM solution in a

volume of 50µl) on a gauze swab over the MMA for 5 minutes, the skull was then washed and dried and topical PGE_2 administered, as above. The effects of the antagonist were also tested following intravenous administration ($3mgkg^{-1}$ in 0.2ml). Here topical application of PGE_2 was carried out as described above with the antagonist given immediately after application of the gauze swab to the closed cranial window.

4.2.4.3 EP_4 antagonist

GW627368 (EP_4 antagonist) or vehicle (1% dimethyl sulfoxide (DMSO), 66% PEG_{200} and 33% distilled water) was also tested topically and intravenously as described above for ZM325802 at a concentration of 2mM and a dose of $0.2mgkg^{-1}$ respectively.

4.2.5 Statistical analysis

Data is represented, where appropriate, as mean ± standard error of the mean, frequency of response or the number of spikes per second. Responses to electrical stimulation of the MMA were normalised as the total response to the train of stimulation directly before treatment and expressed as a percentage of this response. Statistical analysis was carried out using repeated measures ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons (Origin 7 package, Originlab Corporation, USA). $P < 0.05$ was considered significant. Mechanically evoked FRF responses were normalised with respect to control responses and expressed as a percentage of this response. Statistical analysis was carried out using Student's *t*-test (Origin 7 package; $P < 0.05$ was considered significant).

4.3 Results

Data from 92 animals have been included in the analysis. All had acceptable respiratory parameters for the anaesthetised rat and were in the range: pCO_2, 35-45mmHg; pO_2, 90+ mmHg and pH, 7.35-7.45. Data from two animals were not included as O_2 concentration fell below this range. Mean arterial blood pressure (MABP) and heart rate during baseline recordings from all studies was 112 ± 17mmHg and 376 ± 23 beats per minute respectively. Naratriptan caused a small temporary reduction of MABP and heart rate of 11 ± 8mmHg and 15 ± 6, respectively, however this typically lasted no longer than 20 seconds. Intra-arterial infusion of PGE_2, butaprost and vehicle all caused a small increase in MABP and heart rate of 9 ± 4mmHg and 12 ± 5, respectively, which occurred 30 seconds to 2 minutes after the start of infusion and remained elevated for the remainder of the experiment. No significant effect on MABP or HR was seen in other experiments.

All cells responded to electrical stimulation of the MMA with Aδ-fibre latency and a mean response latency of 15 ± 3ms (range: 8.5 to 26.1ms) and a mean amplitude of response of 0.42 ± 0.10mV (range: 0.12 to 0.60mV). The mean threshold of electrical stimulus strength (current amplitude and pulse duration) to elicit neuronal responses of second order neurons in the Vc was 2.9 ± 0.6mA and 0.3 ± 0.06ms respectively, however stimulation at threshold resulted in only one neuronal response in some cells where as other cells showed two or three responses.

4.3.1 Topical PGE_2 application to MMA

For cells tested with PGE_2, electrical stimulation of the MMA during baseline measurements resulted in a mean number of responses to trains of fifty electrical stimulation of 86 ± 14 (n = 8). Following application of 1mM PGE_2 to the area over the MMA, 7/8 cells showed an increased firing rate above baseline. The mean maximum response occurred to the first stimulation train after PGE_2 application where the mean number of spikes was 162 ± 30 (n = 8). However, the onset of the maximum response was variable between cells with 3/8 cells responding with a maximum increase in firing in the first stimulation train after PGE_2 application, 2/8 cells in the second stimulation train and 1/8 cells in the third and fourth train. One of eight cells was unaffected by PGE_2.

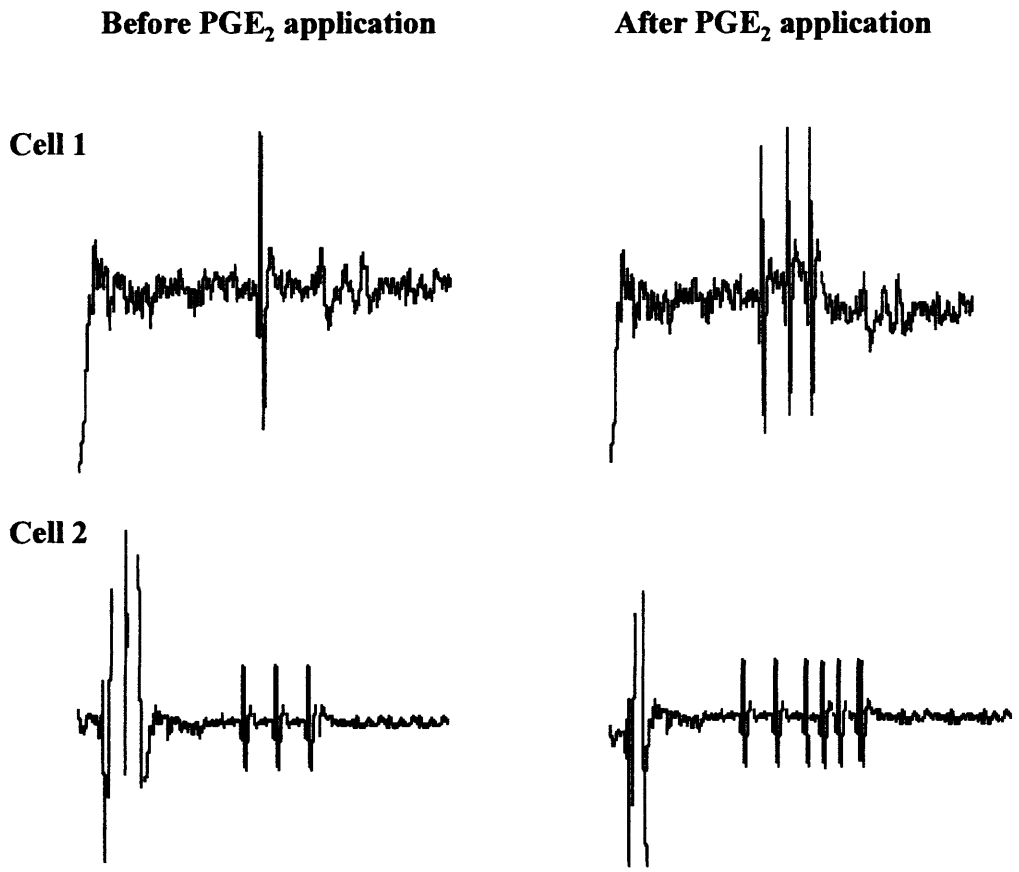


Figure 30 Neuronal responses to electrical stimulation of the MMA, before and after PGE_2 application

Figures show the response of two-second order neurons in the Vc to electrical stimulation of the MMA before and after PGE_2 application. Cell 1 responded to MMA stimulation with latency of 15.5ms and amplitude of 0.38mV; 10 minutes after application of PGE_2, the same cell showed an increased response to an identical stimulus. Cell 2 exhibited three responses to electrical stimulation of the MMA at threshold stimulation levels, the latency of response was in a range of 10.4 to 13.8ms and amplitude of response was 0.21mV; following PGE_2 treatment the same cell typically gave an increased number of responses to the same stimulus.

The application of PGE_2 vehicle to the MMA caused a small increase in the firing rate of Vc neurons in the first stimulation train after application, from pre-treatment values of 79 ± 8 (n = 4; mean number of response to one stimulation train) to 93 ± 4 (n = 4; mean number of response to one stimulation train). However, the mean increase in evoked neuronal activity after PGE_2 treatment (88 ± 15% over pre-treatment levels; n = 8) was significantly greater than that seen following treatment with PGE_2 vehicle (17 ± 2 % over pre-treatment levels; $n = 4$; $P < 0.05$). In 6 cells the increased firing rate that occurred following PGE_2 application returned to baseline levels 30 minutes after PGE_2. However, 1 cell showed an increased firing rate for the remainder of the experiment and 1 cell showed a second phase of increased firing. This "second wave" of increased responses occurred 35 minutes after PGE_2 application and lasted for a further 20 minutes before returning to baseline levels.

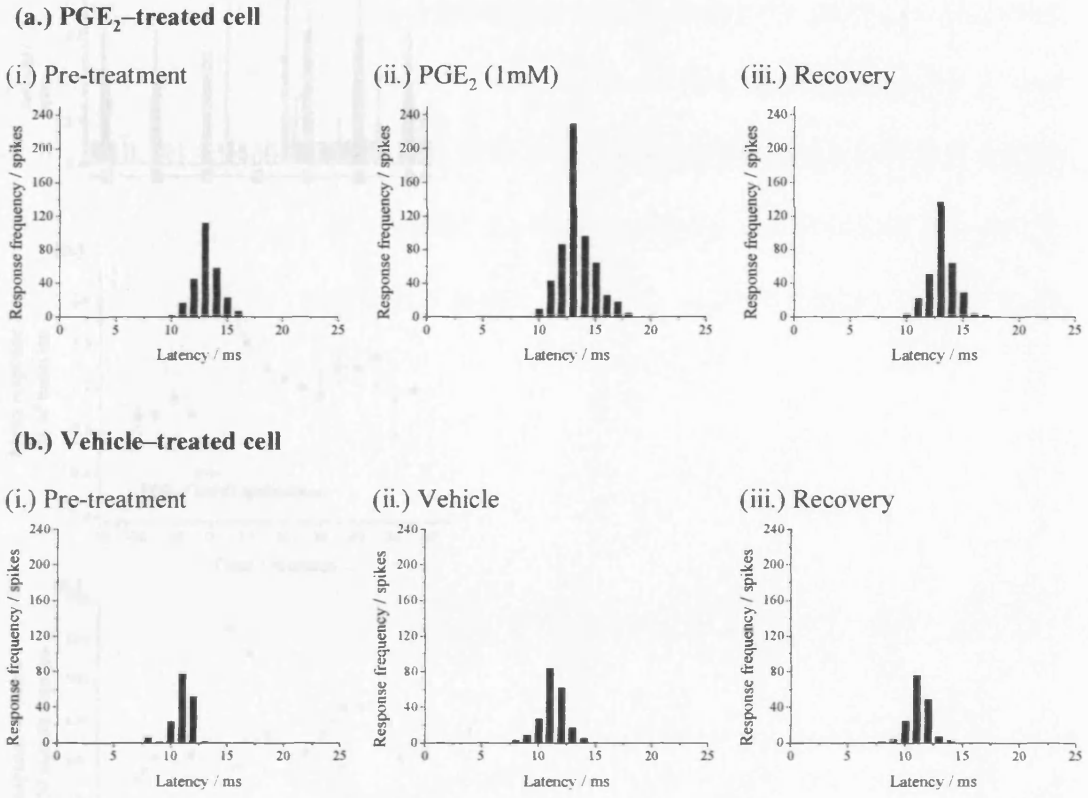


Figure 31 Post-stimulus histograms of electrically evoked responses

Post-stimulus histograms showing examples of single cells responding to electrical stimulation of the MMA before treatment (ai, bi), during treatment with either PGE_2, (1mM; aii) or vehicle, (1% ethanol, 99% saline, bii) and 45-60 minutes after treatment (recovery; aiii, biii). Each histogram represents the frequency of responses occurring at a given latency following electrical stimulus application, taken from 3 stimulation trains (50 stimuli in a single train at 1 Hz) over a 15 minute time period during the indicated treatment phase.

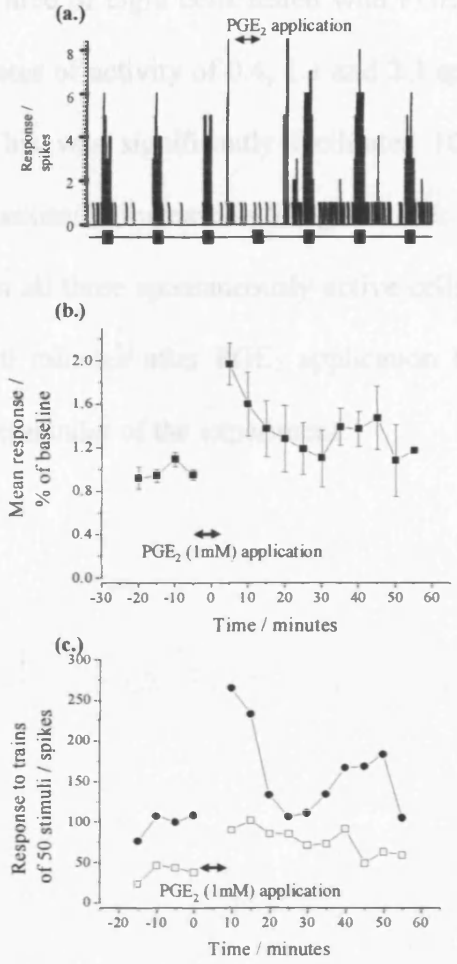


Figure 32 Effect of PGE_2 applied to the MMA on neuronal activity

*(a.) Trace example of a recording of MMA-evoked activity from a single Vc neuron. Blocks at the bottom of the trace represent the occurrence of electrical stimulation trains (50 stimuli at 1 Hz) and individual lines represent the number of spikes recorded at a given time point. Electrical stimulation was turned off during PGE_2 application (marked by arrow). (b) The mean response of all 8 cells tested with PGE_2 application (marked by arrow) are shown. Data is normalised with respect to the total response to the electrical stimulation train given immediately before PGE_2 application and indicates mean ± SEM. A significant increase in MMA evoked neuronal activity which reached a maximum of 88 ± 15% (n = 8) over the maximum increase evoked by PGE_2 vehicle was seen, 5 minutes after PGE_2 application (*P < 0.05; repeated measures ANOVA). (c.) Five of seven cells that showed an increased firing rate following PGE_2 application returned to pre-treatment firing levels 30 minutes after PGE_2. However, 1/7 showed an increased firing rate throughout the experiment and 1/7 cells showed two phases of increased firing which are shown graphically as the mean number of spikes to trains of 50 stimuli.*

Three of eight cells tested with PGE_2 were spontaneously active with mean ongoing rates of activity of 0.4, 1.1 and 2.3 spikes per second during the pre-treatment period. This was significantly facilitated 10 minutes after PGE_2 application with a mean maximum increase in firing of 116 ± 31% over pre-treatment values ($n = 3$; $P < 0.05$). In all three spontaneously active cells, the increased firing rate remained elevated for 20 minutes after PGE_2 application before returning to pre-treatment levels for the remainder of the experiment.

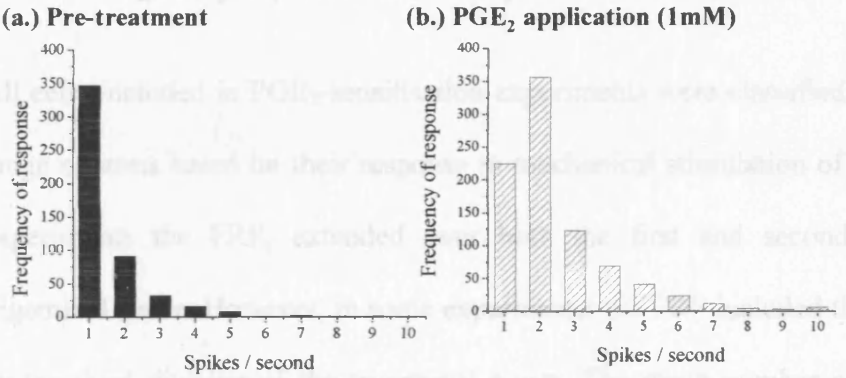


Figure 33 Effect of PGE_2 applied to the MMA on spontaneous neuronal activity

Spontaneous activity of a single Vc neuron. The above figure shows the number of spikes per second, assessed in the 250 seconds between periods of electrical stimulation and displayed as the cumulative total response to three such periods in the 15 minutes preceding PGE_2 application (a) and the 15 minutes immediately after PGE_2 application (b).

4.3.2 Effect of Topical PGE_2 on responses evoked by stimulation of the FRF

All cells included in PGE_2-sensitisation experiments were classified as wide-dynamic range neurons based on their response to mechanical stimulation of the FRF. In most experiments the FRF, extended over both the first and second division of the trigeminal nerve. However, in some experiments the FRF included the area innervated by the third division of the trigeminal nerve. The mean number of spikes resulting from brush and pinch stimulation of the FRF was variable between cells with a range for pre-treatment brush stimulation of 15-53 spikes per 10 second stimulus (n = 24 samples in 8 cells) and a range for pre-treatment pinch stimulation of 19-67 spikes per 5 second stimulus (n = 24 samples in 8 cells). However, all cells showed a significant facilitation to brush and pinch stimulation after PGE_2 application, although this did not reach significant levels until 30 minutes after PGE_2 application. The mean maximum facilitation occurred at a median time point of 60 minutes after PGE_2 application in a range of 30-75 minutes and was 332 ± 103% (n = 24 samples in 8 cells) and 402 ± 102% (n = 24 samples in 8 cells) over pre-treatment levels for brush and pinch stimulation, respectively ($P < 0.05$). PGE_2 vehicle application also resulted in a significant increase in brush and pinch responses over baseline responses of 110 ± 57% (12 samples in 4 cells) and 103 ± 42% (12 samples in 4 cells), respectively ($P < 0.05$). However, the maximum response to PGE_2 vehicle was significantly less than the facilitation seen following PGE_2 application and occurred at a median time point of 30 minutes after application ($P < 0.05$; n = 4).

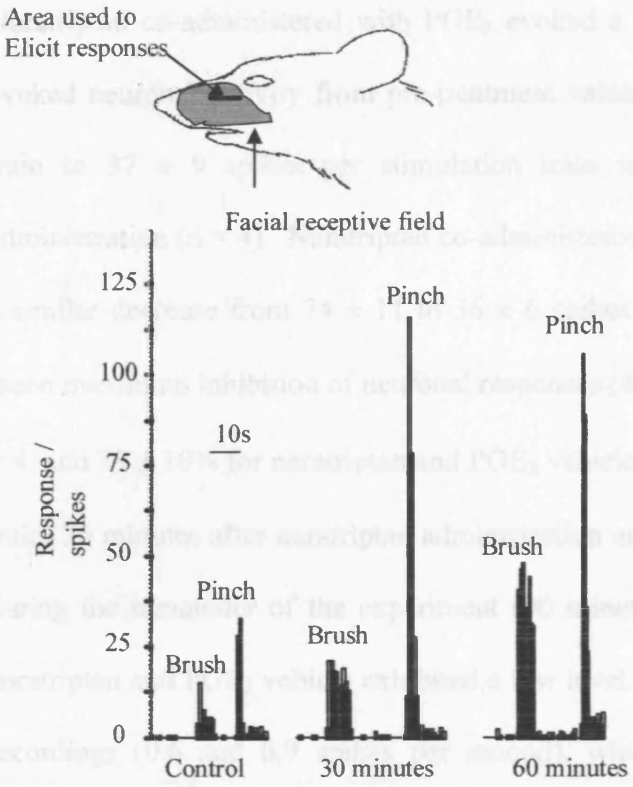


Figure 34 Response to FRF stimulation after PGE_2 application

Neuronal responses of a single Vc neuron activated both by electrical stimulation of the MMA and mechanical stimulation of the FRF. The shaded area of the schematic shows the extent of the FRF and the line indicates the area used to study the response elicited by stimulating the FRF. Traces show the number of spikes elicited following a 10 second brush and 5 second pinch of the FRF before, 30 and 60 minutes after PGE_2 administration to the thinned cranial window over the MMA.

4.3.3 Naratriptan experiments

Naratriptan co-administered with PGE_2 evoked a significant decrease in electrically evoked neuronal activity from pre-treatment values of 78 ± 6 spikes per stimulation train to 37 ± 9 spikes per stimulation train in the first stimulation train after administration (n = 4). Naratriptan co-administered with PGE_2 vehicle (n = 4) evoked a similar decrease from 74 ± 11 to 36 ± 6 spikes per stimulation train (n = 4). The mean maximum inhibition of neuronal responses (85 ± 6% for naratriptan and PGE_2, n = 4, and 79 ± 10% for naratriptan and PGE_2 vehicle) occurred in the fourth stimulation train, 20 minutes after naratriptan administration and responses did not increase again during the remainder of the experiment (90 minutes). Two of four cells tested with naratriptan and PGE_2 vehicle exhibited a low level of ongoing activity during baseline recordings (0.6 and 0.9 spikes per second), which in both cases was completely abolished immediately after naratriptan administration and did not return during the remainder of the experiment. There was no significant difference in the inhibitory effect of naratriptan in animals receiving either PGE_2 or PGE_2 vehicle at any time point ($P > 0.05$). Brush and Pinch responses to stimulation of the FRF were not significantly altered from baseline measurements at any time point in either animals treated with naratriptan and PGE_2 or naratriptan and PGE_2 vehicle. Naratriptan vehicle had no effect on either electrically evoked firing or mechanical stimulation of the FRF compared to pre-treatment responses (n = 3).

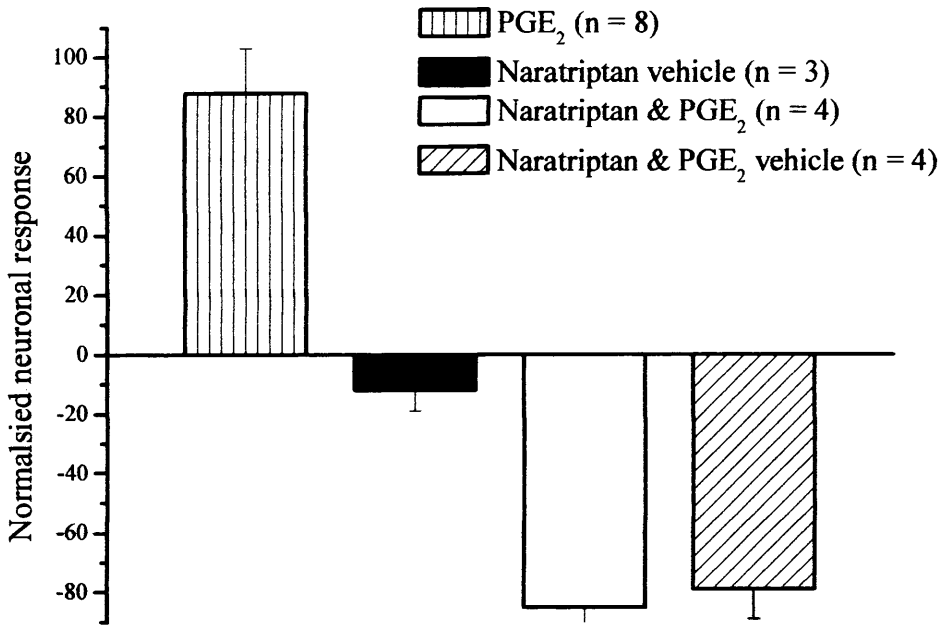


Figure 35 Effect of naratriptan of MMA-evoked responses in the presence of PGE_2

Data represent the total response to the train of electrical stimulation where maximum response was seen normalised to the total response to the electrical stimulation train before drug treatment. Naratriptan co-administered with PGE_2 (n = 4) and Naratriptan co-administered with PGE_2 vehicle (n = 4) evoked a significant decrease in electrically evoked neuronal activity that occurred immediately after administration ($P < 0.05$, repeated measures ANOVA). The maximum inhibition of neuronal responses occurred in the fourth stimulation train, 20 minutes after naratriptan administration and responses did not increase again during the remainder of the experiment (90 minutes). There was no significant difference in the inhibitory effect of naratriptan in animals receiving either PGE_2 or PGE_2 vehicle ($P > 0.05$). Naratriptan vehicle had no significant effect on evoked firing compared to pre-treatment responses ($n = 3$; $P > 0.05$).

4.3.4 Administration of PGE_2 into the FRF

Mean pre-treatment responses to trains of fifty electrical stimulation applied to the MMA were 89 ± 17 (n = 4). Administration of PGE_2 in the FRF did not significantly alter MMA-evoked neuronal firing (maximum change from pre-treatment levels was -16 ± 3%; 75 ± 3 spikes per stimulation train; $n = 4$; $P < 0.05$). There was also no significant effect of PGE_2 vehicle on MMA-evoked neuronal firing (maximum change from pre-treatment levels was -10 ± 4%; 83 ± 8 to 75 ± 3 spikes per stimulation train; $n = 4$; $P > 0.05$). The response to brush and pinch stimulation following PGE_2 or PGE_2 vehicle administration into the FRF was not significantly altered at any time point studied ($P > 0.05$).

4.3.5 Intra-arterial administration of butaprost and PGE2

Mean pre-treatment responses to trains of fifty electrical stimulation applied to the MMA for cells tested with butaprost, PGE_2 and vehicle were 81 ± 9 (n = 3), 75 ± 10 (n = 3) and 71 ± 4 (n = 3) respectively. In the second stimulation train following intra-arterial infusion (10 minutes after the start of the infusion), the mean normalised response of cells treated with butaprost (14 ± 4% below pre-treatment levels; 70 ± 3; n = 3) and PGE_2 (19 ± 3% below pre-treatment levels; 61 ± 11; n = 3) was significantly lower than cells treated with vehicle (14 ± 4% above pre-treatment levels; 81 ± 5; n = 3; $P < 0.05$). However, there was no significant difference in mean normalised responses between butaprost and vehicle for any other stimulation train in the experiment (70 minutes), although there was a slight increasing trend for responses in cells treated with PGE_2, 35 minutes after the infusion but this did not reach significant

levels ($P > 0.05$). The response to brush and pinch stimulation was no significantly altered following infusion of butaprost, PGE_2 or vehicle at any time point studied.

4.3.6 Topical application of butaprost

Topical application of butaprost over the MMA had no significant effect on neuronal firing and the mean responses to trains of fifty electrical stimulation applied to the MMA during pre-treatment recordings and after application of butaprost was 64 ± 14 and 58 ± 6 (n = 5) respectively ($P > 0.05$). The response to brush and pinch of the FRF following topical butaprost application was significantly elevated over pre-treatment responses (72 ± 37% and 49 ± 22% for brush and pinch stimulation respectively) 30 minutes after butaprost. However, this increase was not significantly different to the effect of vehicle and was significantly less than the facilitation induced following PGE_2 application over the MMA ($P < 0.05$).

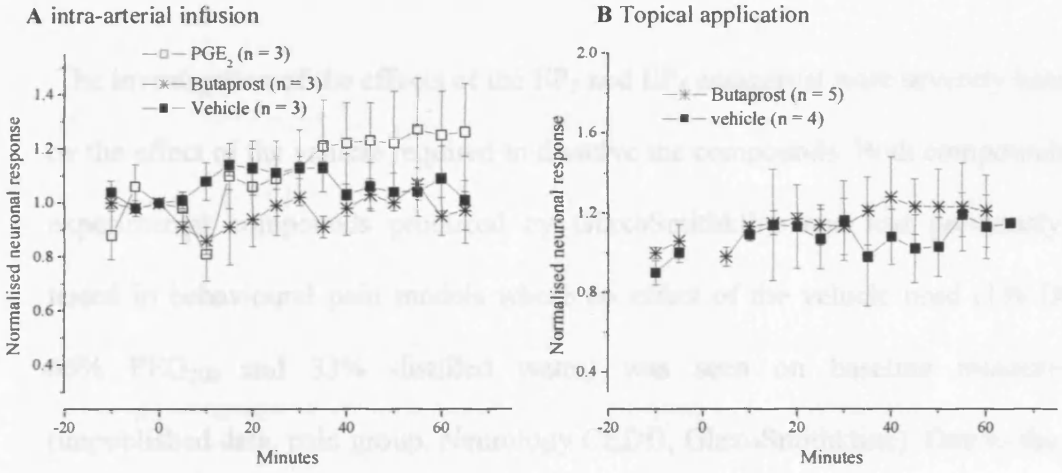


Figure 36 Comparison of the effect of butaprost and PGE_2 following intra-arterial infusion and topical application over the MMA

Electrophysiological recordings were made as described in section 2.1-2.2. Data is represented as the mean normalised response to trains of fifty electrical stimuli applied to the MMA, recorded every five minutes. (a.) Butaprost, PGE_2 or vehicle infusion was started at time 0 for 5 minutes. A significant decrease in neuronal firing was seen, 10 minutes after the start of intra-arterial infusions of butaprost and PGE_2 ($P < 0.05$, repeated measures ANOVA). After this time point, there was no significant difference in the mean normalised response between PGE_2, butaprost or vehicle. (b) Butaprost or vehicle application was made at time 0 for 5 minutes. Butaprost or vehicle had no significant effect on evoked firing compared to pre-treatment levels ($P > 0.05$).

4.3.7 GW627368 and ZM325802

The investigation of the effects of the EP_1 and EP_4 antagonist were severely hampered by the effect of the vehicle required to dissolve the compounds. Both compounds were experimental compounds produced by GlaxoSmithkline and had previously been tested in behavioural pain models where no effect of the vehicle used (1% DMSO, 66% PEG_{200} and 33% distilled water) was seen on baseline measurements (unpublished data, pain group, Neurology CEDD, GlaxoSmithkline). Due to the effect of vehicle on PGE_2 sensitisation in these experiments, a variety of different vehicles were tested, however, only vehicle containing 1% DMSO, 66% PEG_{200} and 33% distilled water was sufficient to allow the compound to dissolve while still leaving a component of the PGE_2 sensitisation seen in earlier experiments.

Intravenous or topically administration of the antagonist vehicle with PGE_2, resulted in a mean maximum facilitation of Vc responses above pre-treatment levels of 36 ± 4% (n = 5) and 37 ± 3% (n = 5) respectively. This was significantly less than the facilitation of 88 ± 15% (n = 8) over pre-treatment levels seen in earlier experiments when PGE_2 was applied over the MMA without co-administration of the antagonist vehicle. However as no facilitation occurred following intravenous or topical administration of the antagonist vehicle with PGE_2 vehicle (mean maximum change of Vc responses relative to pre-treatment levels of -4 ± 5% (n = 5) and 2 ± 6% (n = 5) respectively) a window of PGE_2 sensitisation remained despite the "knock-down" effect of the antagonist vehicle.

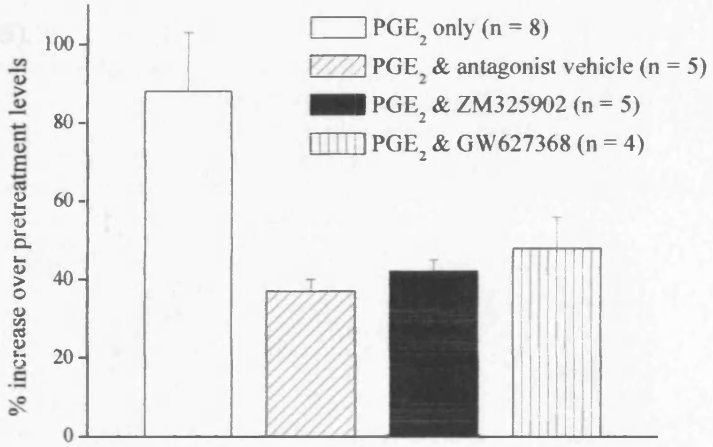
Responses to stimulation in the FRF were also decreased following administration of the antagonist vehicle and PGE_2, compared to PGE_2 alone, with a mean maximum response to brush and pinch of 118 ± 16% and 131 ± 12% respectively (n = 5) after intravenous administration and 154 ± 20% and 133 ± 21% respectively (n = 5) after topical administration. The facilitation of the FRF responses following antagonist vehicle and PGE_2 was not significantly different from the maximum responses observed following administration of PGE_2 vehicle and antagonist vehicle, hence only responses to electrical stimulation of the MMA have been included here.

	Route of antagonist administration	
TREATMENT	**Topical**	**Intravenous**
Antagonist vehicle & PGE_2	37 ± 3% (n = 5)	36 ± 4% (n = 5)
Antagonist vehicle & PGE_2 vehicle	2 ± 6% (n = 5)	-4 ± 5% (n = 5)
GW627368 & PGE_2 vehicle	11 ± 6% (n = 5)	-6 ± 11% (n = 5)
GW627368 & PGE_2	43 ± 8% (n = 4)	34 ± 11% (n = 5)
ZM325802 & PGE_2 vehicle	-11 ± 7% (n = 4)	-16 ± 3% (n = 4)
ZM325802 & PGE_2	43 ± 3% (n = 5)	34 ± 6% (n = 4)

Table 11 Effects of EP_1 and EP_4 antagonists on PGE_2 sensitisation of Vc responses

The above table shows the mean maximum response over pre-treatment Vc firing for the different combinations of PGE_2, PGE_2 vehicle, an EP_1 receptor antagonist (ZM325802), an EP_4 antagonist (GW627368) and the antagonist vehicle. Both intravenous administration and topical application over the MMA of the antagonist was tested. PGE_2 or PGE_2 vehicle was applied topically over the MMA. Application of the antagonist vehicle significantly inhibited the PGE_2 sensitisation of Vc responses compared to the effects of PGE_2 only (see section 4.3.1). The reduced sensitisation seen after PGE_2 and antagonist vehicle was not significantly further reduced by either antagonist following either intravenous or topical application ($P > 0.05$).

(a.) Topical administration



(b.) Intravenous administration

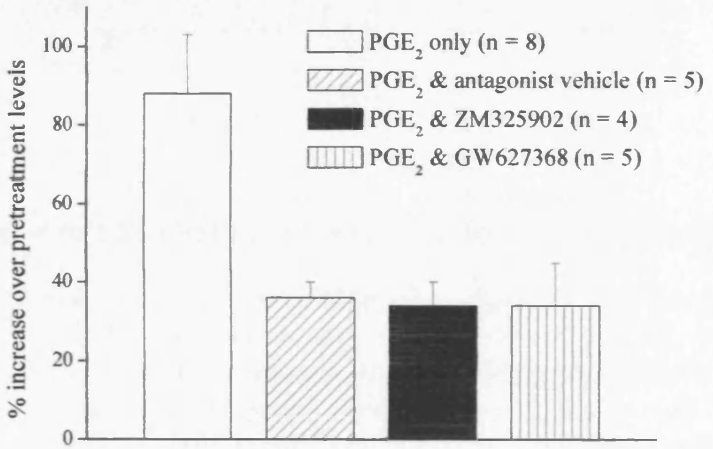


Figure 37 Effect of the EP_1 and EP_4 antagonist on PGE_2 sensitisation

Data represents the maximum normalised effect of PGE_2 relative to pre-treatment responses, applied either alone or preceded by topical application (a) or intravenous administration (b) of antagonist vehicle (1% dimethyl sulfoxide (DMSO), 66% PEG_{200} and 33% distilled water) or the EP_1 or EP_4 antagonist. In all experiments PGE_2 caused it's maximum effect 5-10 minutes after application. Antagonist vehicle significantly reduced PGE_2 sensitisation ($P < 0.05$, repeated measures ANOVA) however, neither antagonist further reduced PGE_2 sensitisation ($P > 0.05$).

Administration of either the EP_1 or EP_4 antagonist by both the topical and intravenous routes had no significant effect on pre-treatment MMA-evoked Vc responses ($P > 0.05$).

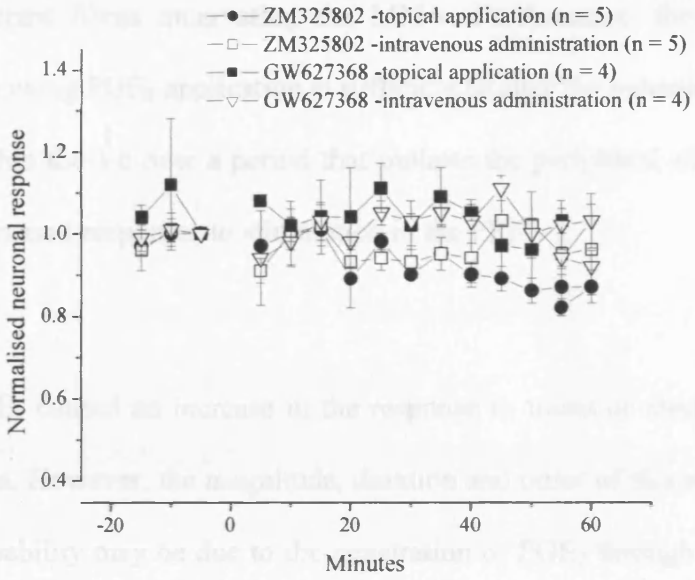


Figure 38 EP_1 and EP_4 antagonists do not effect un-sensitised MMA evoked firing

Electrophysiological recordings were made as described in section 2.1-2.2. Data represents the mean normalised response to trains of fifty electrical stimuli applied to the MMA every five minutes. Antagonists were applied at time 0, as described in section 4.2.4. Neither antagonist, applied topically over the MMA or given as an intravenous dose, had any significant effect on evoked firing compared to vehicle ($P > 0.05$, repeated measures ANOVA).

4.4 Discussion

These results demonstrate that PGE_2 is able to generate sensitisation of dural primary afferent fibres innervating the MMA. Furthermore, the increase in afferent input following PGE_2 application is sufficient to alter the behaviour of second order neurons within the Vc over a period that outlasts the peripheral stimulation, evidenced by the increased responses to stimulation in the FRF.

PGE_2 caused an increase in the response to trains of electrical stimuli applied to the dura. However, the magnitude, duration and onset of this response were variable. This variability may be due to the penetration of PGE_2 through the closed cranial window, as although the area of the window overlying the branch of the MMA could be reproduced accurately, the thickness of the window could not be measured under these experimental settings. Alternatively, the variability may have been a result of the differing thresholds of peripheral neurons; some of which may have been unavoidably sensitised by the mechanical disruption of drilling a cranial window. To control for this, further experiments where an open cranial window is used and PGE_2 is applied directly to the dura mater could be carried out. However the advantage of the increased reliability in the response may be outweighed by the disadvantages of a craniotomy e.g. release of inflammatory mediators following removal of the dura mater that is strongly adhered to bone.

The increase in spontaneous activity in this study is also interesting as it is suggestive of a possible direct activation of neurons by PGE_2. Despite previous reports of direct

effects on membrane potential, spontaneous activity, calcium currents and neuropeptide release in sensory neurons (Evans *et al.*, 2000; Nicol *et al.*, 1992; White *et al.*, 1996), PGs have long carried a dogma of being sensitising agents only, with no ability to activate neurons directly. A recent study (Smith *et al.*, 2000) confirms the ability of PGE_2 (1-10μM) to directly activate DRG neurons in culture, as well as facilitating the effects of bradykinin. The authors suggest a direct sensitising effect of prostaglandins is often not seen in other *in vitro* experiments due to a low efficacy at room temperature. While it is also possible that in these studies, the increase in spontaneous activity is the result of a sensitising action of PGE_2 to an endogenous dural substance, present under resting conditions at an insufficient concentration to cause activation of primary afferents alone, a direct activating effect of PGE_2 should not be ruled out.

Off-line analysis of spontaneously active cells in this study was conducted to find any correlation between pulsatile blood flow and peaks in neuronal firing after sensitisation with PGE_2. Mean heart rate was unaltered by PGE_2 application (396 ± 9 beats/ minute (n=8), typically 1 beat every 0.15 seconds), hence if a correlation was occurring, a peak in neuronal firing should be evident approximately every 0.15 seconds. However, no correlation was evident in any of the spontaneously active neurons in this study at any time point after PGE_2 application. Therefore, it is unlikely that PGE_2 sensitises peripheral terminals innervating the MMA to a degree where normal blood flow is sufficient to cause activation of sensory neurons, a theory that has been suggested to explain the throbbing nature of the migraine pain and the efficacy of vasoconstrictors in migraine (Yamamura *et al.*, 1999).

The sensitisation to light brush and noxious pinch of the FRF occurred maximally 60

minutes after the PGE_2 stimulation, which corresponds well to clinical data showing allodynia on the ipsilateral side of the face occurs approximately 1hour after the start of a migraine (Burstein *et al.*, 2000). This increased response to facial stimulation is likely to be a reflection of lowered thresholds in second order neurons that receive convergent inputs from facial tissues and the dura. It is of interest to note that the facial hypersensitivity in this study was not seen until 30 minutes after PGE_2 application when the increased response to MMA stimulation had ended. This implies that while a peripheral input is necessary to initiate central changes, the maintenance is independent of an ongoing peripheral input. This is in good agreement with other studies where a brief input from primary afferents have been shown to alter dorsal horn neuronal activity over a significant period of time by shifting the membrane potential towards a more depolarised state (Woolf 1983). However, given that the response to noxious pinch of the FRF as well as the response to light brush of the FRF was still elevated after 1 hour, it is surprising that the response to noxious, peripheral MMA stimulation was not also still elevated at this time point. The difference in the sensitisation response from the two areas, may therefore represent a difference in central modulation of cutaneous and visceral inputs at the level of the Vc, a concept which has been further explored in study 3.

While the effects of PGE_2 in this study are promising in the development of a model of *in vivo* dural sensitisation, in order to investigate the effects of PGE_2 in a physiological system, further model development is required. One such development could involve the chronic implantation of a cannula over the dura around the MMA, which would allow the effects of PGs to be tested when any sensitisation caused by the surgery had subsided. Previous animal studies have shown that dural-evoked Vc responses can be

inhibited following stimulation of the PAG (Knight & Goadsby, 2001) or enhanced following blockade of P/Q-type calcium channels in the PAG (Knight *et al.*, 2002). Hence, it would be interesting to look at PGE_2-induced sensitisation in animals where disruption of descending pathways from supraspinal structures such as the PAG had occurred, in order to assess whether sensitisation is greater following descending pathway disruption. This type of study may provide insight into the possibility that migraineurs have a genetic susceptibility to the effects of agents acting peripherally due to inefficiency of descending inhibitory controls from regions such as the PAG.

It is unlikely that application of PGE_2 other than directly to the sites of interest will result in a measurable sensitisation, evidenced by the lack of effect of PGE_2 in generating sensitisation following intra-arterial infusion. However, given the effects seen following PGE_1 infusion into migraineurs, a generalised increase in circulating PG level cannot be ruled out, and may be related to dose and duration of infusion. It is also true to say, that an increase in Vc activity cannot, with current methods, be directly correlated with headache scores in humans. Therefore, the effects of PGs should also be assessed in other *in vivo* animal models of migraine, e.g. the vasodilator effects of prostaglandins are well known, although there have been no studies that have looked at the direct vasodilator effect on the MMA. This could possibly be determined in the intravital model allowing the time course of any dilatation to be correlated with effects on neuronal activity and hopefully highlight any possible relationship of abnormal dilatation and enhanced neuronal activity.

The inhibitory effects of naratriptan that were similar in both the presence and absence of PGE_2 is in keeping with a recent study which found that in a rat model, triptans

prevented the induction of sensitization in central trigeminovascular neurons (recorded in the Vc), but not in peripheral trigeminovascular neurons (recorded in the trigeminal ganglion) (Levy *et al.,* 2004). After sensitization was established in both types of neuron, triptan administration effectively normalized intracranial mechanical sensitivity of central neurons, but failed to reverse such hypersensitivity in peripheral neurons. In agreement with the rationale for the use of triptans in this study, the authors (Levy *et al.,* 2004) proposed;

> "In the early stages of migraine, central trigeminovascular neurons become activated in response to incoming impulses from meningeal nociceptors . At this stage, presynaptic inhibition by triptans should be sufficient to render the central neurons quiescent, resulting in a pain-free state. In migraine attacks associated with cutaneous allodynia, the central neurons eventually become sensitized and develop their own autonomous activity. At this stage, the same presynaptic inhibition by triptans becomes progressively inadequate to render central trigeminovascular neurons quiescent, resulting in only partial pain relief. In migraine attacks that remain allodynia-free, the central trigeminovascular neurons do not reach a state of sensitization and continue to be driven by incoming impulses from meningeal nociceptors. Therefore, the central presynaptic inhibition exerted by triptans should be sufficient to render the patient pain-free at any time during the attack".

Responses to facial stimulation following naratriptan administration in this study remained unaffected throughout the experiment, lending weight to the theory that an increased peripheral stimulation is required to alter the activity of central neurons.

The investigation of the EP receptor responsible for PGE_2 sensitisation in this model,

in the first instance, showed that an action through the EP_1, EP_2 or EP_4 receptor is unlikely to solely mediate the effect. However, the study was unable to rule at the possibility that PGE_2 acts through more than one EP receptor to exert its effect. Furthermore, the study also showed that the EP_1 or EP_4 receptor are unlikely to contribute to the evoked firing elicited by MMA stimulation in the un-sensitised state.

The modulation by EP receptors of un-sensitised MMA evoked responses is of interest in respect to results reported by Ellrich and colleagues (Ellrich *et al.* 1999). In that study, the non-steroidal anti-inflammatory drug, acetylsalicylic acid (ASA), given as a intravenous dose or applied topically to the dura mater and caused a significant reduction of second-order neuron activity recorded in the Vc, responding to mechanical stimulation of the dura mater, in the area of the MMA. While these results indicate an involvement of PGs in evoked trigeminal firing, it is not necessarily due to the effects of PGE_2 and may well be an effect of other functional PGs, such as PGI_2 or PGD_2. A further caveat in comparing the results of this study, with the experiments described in this thesis, is the variation in methodology. In the model used by Ellrich and colleagues, a full craniotomy was performed, which may have resulted in the production of an inflammatory response *per se*. In addition, while they used electrical stimulation of the MMA to identify dural responsive cells within the Vc, the experimental protocol used mechanical stimulation of the dura mater, which may well activate a different subset of dural afferents compared to electrical stimulation.

As ASA is extremely effective in the treatment of migraine clinically, especially when given intravenously, understanding its mechanism of action within the trigeminal system is likely to provide a vital insight into the pathological processes occurring

during migraine. However, this may be related to a central effect and as such it is interesting to note that when given intravenously, but not topically in the above animal model, a significant reduction of spontaneous Vc activity was seen (Ellrich *et al.*, 1999). It should also be remembered, that while the inhibitory effects of ASA on PG production are well known, it is also possible that ASA may have an effect on other signalling cascades.

The investigation of the role of EP_1 and EP_4 receptors in dural PGE_2 sensitisation in this study was hampered by vehicle effects, with a significant reduction of the PGE_2 sensitisation seen when vehicle was co-administered. This is likely to be the result of an effect of either PEG_{200} or DMSO as there are several sensitisation studies using compounds administered in water, with no vehicle effects. As vehicle had no effect on MMA-evoked firing in the control situation, it is likely that PGE_2 results in activation of an intracellular cascade that is selectively inhibited by vehicle. While this modulation by vehicle could equally be due to the effects of PEG_{200} or DMSO, there are no reported effects of this type of effect for PEG_{200}, however several others have reported similar decreased sensitisations by DMSO (van't Erve *et al.*, 1998).

While the reduction of sensitisation by vehicle in this model was considerable, a significant window of sensitisation of up to 40% over pre-treatment levels remained. Hence, it is worth considering that investigation of the effects DMSO may provide insight into a common final non-specific mechanism of neuronal sensitisation following initiation of sensitisation by different receptor pathways. However as DMSO is highly lipophilic and rapidly absorbed through skin, it is likely to have widespread effects throughout the body and this is supported by the similar effects on

PGE_2 sensitisation seen following both intravenous and topical vehicle administration.

Due to the vehicle effects, the results of these studies are not conclusive and an effect of the EP_1 and EP_4 antagonists may have been "hidden". Although as either antagonist failed to reduce the remaining sensitisation, it is unlikely that an action through either of these receptors is entirely responsible for the sensitisation and to rule out an effect at several receptors, a combination of antagonists needs to be assessed.

The lack of effect of the EP_2 agonist, butaprost, in this model is slightly surprising as in an *in vitro* preparation of trigeminal ganglion neurons, butaprost was almost as effective as PGE_2 in causing CGRP release (Jenkins *et al.*, 2001). However, while the presence of EP_2 mRNA in trigeminal ganglion cells has been confirmed, EP_2 receptor expression has not been examined. Within the systems that have been studied, EP_2 is largely thought to be induced following inflammatory stimulation, hence the process of neuronal culturing, where cells are subjected to considerable mechanical disruption, may be the factor responsible for inducing EP_2 receptor expression. Accordingly, the role of the EP_2 receptor in trigeminal signalling with relevance to migraine is not easily assessed at present as it is not currently known to what extent a dural neurogenic inflammation is part of the pathological events occurring during migraine. If a dural inflammation where to occur, it is possible that the antagonists at the EP_2 receptor may provide an attractive target for acute treatment.

It is also possible that PGE_2 may also be acting through IP or DP receptors as a degree of cross talk between the receptors has previously been suggested (Kiriyama *et al.*, 1997). If this were the case it may also be interesting to look at PGI_2 or PGD_2 induced

sensitisation, or the effects of an IP or DP antagonist against PGE_2 sensitisation.

In summary, the results in this study support the hypothesis that peripheral dural sensitisation generates an altered central neuronal activity that may underlie some of the clinical features of migraine such as head pain and facial skin hypersensitivity. While this study does not provide evidence for an endogenous source of prostaglandins in the development of dural sensitisation, the time course of sensitisation following application of PGE_2, which is in agreement with that reported to occur following the onset of a migraine attack merits consideration of prostaglandins as an endogenous mediator of migraine. Hence, further investigation into the mechanism of action of prostaglandins in migraine, may not only lead to the development of a successful pre-clinical migraine model but may also generate new treatment strategies against migraine pain.

5 Study 3-Exploration of the characteristics of Vc neurons with relevance to primary afferent input

5.1 Introduction

Due to the anatomical similarities between the spinal dorsal horn and Vc, comparisons are often made regarding the mechanisms of responses to nociceptive stimulation in both systems. However, previous studies have shown fundamental differences in the responses of Vc neurons compared to those reported in the dorsal horn (Bereiter *et al.*, 2000; Cumberbatch *et al.*, 1998). For example, the threshold of activation for C-fibre evoked responses has been reported to be significantly higher for VBNC neurons (between 6-8mA and up to 2ms,) compared to lumbar neurons (3-4mA; Bouhassira *et al.*, 1987). Furthermore, diffuse noxious inhibitory controls have also been found to be more pronounced for trigeminal neurons than lumbar neurons and the mean discharge of trigeminal neurons has also been suggested to be less than those recorded at the lumbar region in similar experimental conditions (Villanueva *et al.*, 1985, Dickenson *et al.*, 1980).

Due to the trigeminal system's dual representation, it has also been shown that peripheral stimulation of the cornea results in activation of neurons in the VBNC in two distinct areas, namely the Vi /Vc border and the Vc / C_1 border, which respond differently to the same stimulation (Meng *et al.*, 1997). Moreover, as discussed in the previous chapter, the anti-migraine triptan drugs appear to specifically inhibit Vc responses and not dorsal horn response to noxious stimulation (Cumberbatch *et al.*, 1998). Hence, while the trigeminal system is capable of generating responses that are comparable to those of the dorsal horn, the highly convergent nature of inputs to the

Vc and the modulation they receive are poorly understood, especially in relation to the pathology of primary headache syndromes.

A further complication of the interpreting the response of Vc neurons to dural stimulation, is that this type of stimulation may be considered as a visceral input. Until the last decade or so, it was thought that data regarding nociceptive processing from cutaneous regions could be extrapolated to include the viscera. However, there are fundamental differences between pain arising from cutaneous and visceral areas, for example, mechanical trauma is an ineffective stimulus for visceral pain but highly effective for cutaneous areas. Visceral pain is also poorly localised, with secondary hyperalgesia evident at sites remote from the source compared to cutaneous pain, which is normally highly localised with considerable secondary hyperalgesia at the site of damage Additionally, visceral pain is associated with autonomic disturbances, which is a prominent feature of many migraine attacks. (for review see McMahon *et al.*, 1995).

A further fundamental difference between sensory and visceral neurons is their ability to exhibit the electrophysiological phenomenon of "wind-up". As described in section 1.7.1., in somatic sensory neurons, the number of neuronal responses to a fixed stimulation increases throughout a stimulation train applied to a peripheral nerve. However, while visceral neurons do exhibit increased excitability following repetitive stimulation (Laird *et al.*, 1995) they appear unable to "wind-up". Furthermore, the relay of information to the central nervous system also varies between the somatic and visceral systems. Until recently, it was assumed that all nociceptive signals, visceral and somatic, ascended to the brain via the spinothalamic tract. However, the discovery

of the dorsal column pathway, the spino (trigemino)-parabrachio-amygdaloid pathway and the spinohypothalamic pathway all of which carry visceral nociceptive information have added a new level of complexity to the dissection of higher order integration (Al-Chaer *et al.*, 1988; Jasmin *et al.*, 1997; Katter *et al.*, 1996). The physiological basis for the differences between cutaneous and visceral pain is not clear, although there is some evidence that primary afferents innervating visceral structures form a discrete class of sensory receptors that fundamental differ in their activation properties from cutaneous receptors (Cervero & Laird, 1999; Gebhart, 1995). A nociceptive specific type, an intensity coding type with a low threshold to natural stimuli and a group of "silent receptors" that only respond to stimuli in the presence of inflammation have all been proposed. As such, the investigation of the properties of dural afferents and the central neurons they synapse with is a crucial step in the investigation of migraine pathology.

During migraine, a considerable degree of central sensitisation may be present in the Vc, with many resultant neuroplastic changes in the "wiring" of neurons. This may result from dural inflammation (Moskowitz, 1992), or altered descending pain modulation (Goadsby *et al.*, 1991), or both, and significantly affect the behaviour of Vc neurons. Certainly, under conditions of peripheral inflammation, wind-up of dorsal horn is enhanced, not only in the inflamed area but also when the stimulation is applied to adjacent areas (Herrero *et al.*, 1996; Stanfa *et al.*, 1992), with saturation of the wind-up response occurring significantly later than in control animals, indicating an increased maximum firing rate of neurons. Hence, in this study, mustard oil (MO), a well known C-fibre activator (Woolf & Wall, 1986; Reeh *et al.*,1986) has been applied to the face to induce central sensitisation within the Vc, and the wind-up

response further examined.

5.2 Methods

Electrophysiological recording of second order neurons in the Vc responding to electrical stimulation of primary afferents innervating the MMA and face, with both Aδ- and C-fibre latency were carried out as described in section 2.1.

5.2.1 Wind-up of Vc cells using dural stimulation

Neuronal identification was carried out with a search stimulus of 0.8ms, 6mA at 1Hz. Higher stimulus parameters were used than in the study 1 or 2 to elicit C-fibre responses as well as A-δ responses. Once a neuron in the Vc responding to dural stimulation had been identified, cells were characterized, by latency of response (i.e. A-δ or C-fibre input) and type (nociceptive specific, wide-dynamic range or low threshold). Where a FRF could be found, single unit recordings were made in response to light brush of the FRF by monitoring the response to a 10 second brush of the area and repeated three times to ensure reproducibility and to permit statistical analysis between individual cells. The preparation was then left for 10 minutes to minimise sensitisation from FRF stimulation before the electrical stimulation protocol was started.

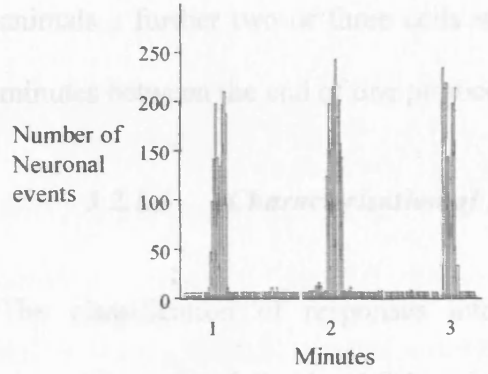


Figure 39 Brush responses of the FRF

Typical neuronal control response recorded in the Vc following brush stimulation of the FRF. Each stimulation was for a ten second period and repeated three times to ensure reproducibility.

Two electrical stimulation protocols were tested as follows:

1) 1 Hz, (1.5 X threshold X 20), repeated every 3 minutes for 30 minutes

2) 2 Hz, (1.5 X threshold X 20), repeated every 3 minutes for 30 minutes

Although other studies have typically used electrical stimulation parameters of 2ms pulses at 3 times C-fibre threshold at between 0.66 Hz and 1 Hz (Dallel *et al.*, 1999) to generate wind-up, in our experimental set-up, we have found that electrical stimulation pulses over 1 ms, recruit additional neuronal responses. Furthermore, we have also found that electrical stimulation at frequencies lower then 1Hz fail to produce reliable responses to electrical stimulation. This may be as a result of stimulating through a closed cranial window, such that there is dissipation of the current which would hence reduce the intensity of stimulation of primary afferent fibres. At the end of each

stimulation protocol, FRF characterisation was repeated as described above. In some animals a further two or three cells were examined with a rest period of at least 30 minutes between the end of one protocol and start of the next.

5.2.1.1 Characterisation of Aδ and C-fibre responses

The classification of responses into Aδ and C-fibre latencies were based on observations that following MMA stimulation, two distinct groups of responses were seen. The first group of responses appeared 7-25 ms after stimulation, attributed to A-δ fibre input, and the second group appeared 40-80 ms after stimulation, attributed to C-fibre input. With an estimated conduction distance of 25 mm, and assuming a time of 1 ms for synaptic transmission, a range of conduction velocities of 1.0-4.0 ms^{-1} for A-δ responses and 0.30-0.65 ms^{-1} for C-fibre responses was observed, in general agreement with standard definitions (Guyton & Hall, 1996).

5.2.2 Wind-up of Vc cells using facial stimulation

Due to the problem of muscle contraction seen following electrical stimulation of the face, the neuromuscular blocking agent, vecuronium bromide, was administered in these experiments as described in section 2.1. Neuronal identification was carried out as the electrode was advanced through the Vc by lightly stroking the face and vibrissae, until a response was seen. The cell was then examined for response to noxious stimulation by application of pinch and pressure, and only those cells fulfilling the criteria of a wide-dynamic range (WDR) cell were examined (Hu *et al.*, 1981). For electrical stimulation, bipolar needle electrodes were inserted into the facial skin and secured with tape. A 1 Hz electrical stimulation protocol, as described for

wind-up with dural stimulation was used for all cells in this study.

5.2.3 The effect of MK-801, an NMDA antagonist, on MMA-evoked Vc responses

Following identification of a Vc neuron linked to MMA stimulation, an electrical simulation protocol (1Hz, 1.5 threshold X 50, repeated every 5 minutes) was started and following establishment of a steady baseline (< 10% variation), over at least three stimulation trains. The NMDA receptor antagonist, MK-801 ($1mgkg^{-1}$) or vehicle (saline) was then given intravenously in 0.2ml and electrophysiological recordings to MMA stimulation continued as above.

5.2.4 Wind-up of Vc cells following the induction of central sensitisation

Following identification of a Vc neuron linked to MMA stimulation, the size of the FRF was estimated using brush stimulation and the response to a 10 second brush stimulation was the carried out as described for the wind-up protocols above. Noxious pinch stimulation was not assessed in these experiments, as previous observations had shown that while the magnitude of the response to noxious stimulation remains constant for several hours, considerable variation in the size of the FRF occurs following noxious stimulation, which is in agreement with data form Dubisson *et al.*, (1979). As MO was applied outside the FRF in these experiments, it was hence of interest to follow the size of the FRF.

Following FRF characterisation, an area of the face was then shaved (approx. $1cm^2$) adjacent to the FRF to facilitate topical MO application. An electrical simulation protocol (1Hz, 1.5 threshold X 50, repeated every 5 minutes) was then started and

following establishment of a steady baseline (< 10% variation), over at least three stimulation trains, MO (10% in mineral oil) or vehicle (mineral oil) was applied to the shaved skin. The response to FRF stimulation was then re-examined 30 and 45 minutes after MO application.

5.2.4.1 Data analysis

Data is presented as mean ± standard error of the mean. Wind-up for Aδ and C-fibre responses were assessed separately. The neuronal response to individual stimulation trains were examined using logistic regression analysis (Origin 7 software package) to determine any increase in response from the first to the last stimulation in the train, where a significant regression ($P < 0.05$) was regarded as an indication of wind-up. A between train effect was then compared using a repeated measure analysis of variance (ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons, Origin 7 software package) where $P < 0.05$ was regarded as significant. The effect of FRF before and after wind-up protocols was also compared using a repeated measure ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons where $P < 0.05$ was regarded as significant. For MO experiments, responses to electrical stimulation of the MMA were normalised as the total response to the train of stimulation directly before treatment and expressed as a percentage of this response. Statistical analysis was carried out using repeated measure ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons (Origin 7 package, Originlab corporation, USA). $P < 0.05$ was considered significant.

5.3 Results

Single unit recordings were made from 46 Vc neurons in 27 animals. Data from 2 animals treated with MO, were not included in the quantitative analysis as cells were lost directly after MO application.

5.3.1 Wind-up of cells receiving a dural input

Electrophysiological recordings were made from 17 cells in the Vc responding to electrical stimulation of the MMA in eight rats. All had acceptable respiratory parameters for the anaesthetised rat: pCO_2, 35-45 mmHg; pO_2, 90+ mmHg and pH, 7.35-7.45. Mean arterial blood pressure (MABP) and heart rate were 105 ± 7 mmHg and 382 ± 6 beats/minute, respectively. Cells were located in the depth range 350-1200 μm below the pial surface. Five of seventeen cells exhibited an Aδ- response only, 4/17 a C-fibre response only, and 8/17 both an Aδ and C-fibre component. Average response latencies and dural stimulus threshold (current and pulse duration) were 14 ± 2 ms, 2 ± 0.6 mA and 0.3 ± 0.04 ms for Aδ, and 45 ± 7 ms, 6 ± 1.5 mA and 0.8 ± 0.1 ms for C-fibres. In one cell in this dural stimulation group, C-fibre input only, a FRF could not be found, whereas all other cells studied showed a convergent input from the face, assessed by non-noxious stimulation.

5.3.1.1 Aδ response

Of the 13 cells responding to MMA stimulation with A-δ fibre input latency, 6 were tested with the 1 Hz protocol (2 Aδ only and 4 Aδ + C-fibre) and 7 were tested with the 2 Hz protocol (3 Aδ only and 4 Aδ + C-fibre). For the 1Hz protocol, the mean

number of spikes to the first and last stimulus in the first stimulation train were not significantly different (1.5 ± 0.3 and 1.7 ± 0.4 respectively; n = 6; $P > 0.05$). This lack of wind-up was observed in all stimulation trains in the experiment and the mean number of spikes to the first and last stimulus in the train in the last stimulation train of the protocol were not significantly different to those in the first stimulation train (1.4 ± 0.5 and 1.6 ± 0.3 respectively; n = 6; $P > 0.05$).

The number of spikes per stimulus was significantly greater during the 2Hz protocol than the 1 Hz protocol, however there was no significant wind-up effect observed in any cell. In the first stimulation train of the protocol, the mean number of spikes to the first and last stimulus in the train was 2.8 ± 0.8 (n = 7) and 3.1 ± 0.7 (n = 7) respectively. As for the 1Hz protocol, this was not significantly different for any stimulation trains in the experiment and the mean number of spikes to the first stimulation and last stimulus in the last stimulation train was 2.7 ± 0.5 (n = 7) and 2.9 ± 0.6 (n = 7) respectively ($P > 0.05$). In either the 1 Hz or 2 Hz protocol there was no significant difference between the number of spikes per stimulus for cells that showed an Aδ-fibre component only compared to cells that showed both an Aδ and C-fibre component.

5.3.1.2 C-fibre response

Of the 12 cells responding to MMA stimulation with C-fibre input latency, 6 were tested with both the 1 Hz and 2 Hz protocol (2 cells with C-fibre only response and 4 cells responding with both A-δ and C-fibre latency in each group). As for Aδ-fibre mediated responses, C-fibre responses showed no significant wind-up when tested

with either the 1 or 2 Hz protocol. For the 1Hz protocol, the mean number of spikes to the first and last stimulus in the first stimulation train was 0.9 ± 0.2 and 1.1 ± 0.3 respectively (n = 6), which was not significantly different for any stimulation train in the experiment ($P > 0.05$). For the 2Hz protocol, the mean number of spikes to the first and last stimulus in the first stimulation train was 1.4 ± 0.5 and 1.7 ± 0.6 respectively (n = 6) which again was not significantly different for any stimulation train in the experiment ($P > 0.05$).

5.3.1.3 Brush-pinch responses

In most experiments the FRF, extended over both the first and second division of the trigeminal nerve, however, in two experiments, the FRF included the area innervated by the third division of the trigeminal nerve and in one experiment, no FRF could be found. Responses to brush of the FRF varied considerably between cells with a range of 17 to 312 spikes per 10 second brush stimulation (range observed from 96 brush stimulations in 16 experiments). However, there was no significant difference between the response to FRF stimulation before the start of the electrical stimulation protocol and that at the end of the electrical stimulation protocol for individual cells ($P > 0.05$).

5.3.2 Wind-up of cells following electrical stimulation of the FRF

Electrophysiological recordings were made from 18 cells in the Vc responding to electrical stimulation of the face and non-noxious stimulation of the face in eight rats. Eight cells showed responses consistent with the latency of A-δ fibre input only, five cells responded in the C-fibre range and five cells showed both an early and late response attributed to A-δ and C-fibre mediated inputs, respectively. In all

experiments, infusion of neuromuscular block had a gradual depressant effect on MABP and HR. The mean MABP and HR at the start of neuromuscular block infusion was 112 ± 11 mmHg and 390 ± 18 beats per minute, respectively, compared to two hours after the start of infusion, where mean MABP and HR were 89 ± 8 mmHg and 327 ± 23 beats per minute, respectively.

5.3.2.1 A-δ responses

Of the eight cells responding to electrical stimulation of the face with A-δ latency only, four cells tested were recorded in the superficial layers and four in the deeper layers of Vc, with a mean recording depths of 236 ± 43 μm and 704 ± 48 μm respectively below the pial surface. The five cells tested that demonstrated both A-δ and C-fiber components were all located in deeper layers of the Vc, with a mean recording depth of 925 ± 57 μm. No significant difference in the stimulation parameters required to elicit responses were seen between cells recorded in superficial (7.1 ± 1.1 mA, 0.8 ± 0.1 ms) and deep layers (6.9 ± 1.3 mA, 0.9 ± 0.2 ms). In cells that exhibited both an A-δ and C-fiber component, stimulation parameters required to elicit the A-δ component of the response was not significantly greater than to elicit responses in cells responding with A-δ latency only (7.9 ± 1.5 mA, 1.0 ± 0.3 ms), however, stimulation parameters generally had to be increased to see reproducible C-fiber responses ($P < 0.05$). All cells responding with A-δ fiber latency, showed no significant wind-up in any stimulation train and the mean number of spikes to the first and last stimulus in the first stimulation train was 1.9 ± 0.6 and 2.2 ± 0.5 (n = 12) respectively (n = 12) which was not significantly different in any stimulation train in the experiment ($P > 0.05$). However, one cell did show a small increase in response

with successive stimulations in the last three stimulation trains only, however the increase did not reach significant levels ($P = 0.074$).

5.3.2.2 C-fibre responses

All cells responding to electrical stimulation of the FRF with C-fibre latency were recorded from the deeper layers of Vc with a mean recording depth of 1026 ± 83 μm below the pial surface. Stimulation parameters required to elicit C-fibers were of greater duration than those required to elicit A-δ responses with mean parameters of 9.2 ± 1.8 mA and 1.7 ± 0.4 ms ($n = 10$). Six of ten C-fibers showed a significant wind-up response to electrical stimulation of the face ($P < 0.05$) while the remaining four cells showed no significant wind-up ($P < 0.05$). Of the cells showing a wind-up response, the mean number of spikes to the first and last stimuli in the first stimulation train of the protocol was 1.8 ± 0.4 and 6.3 ± 0.3 respectively ($n = 6$; $P < 0.05$).

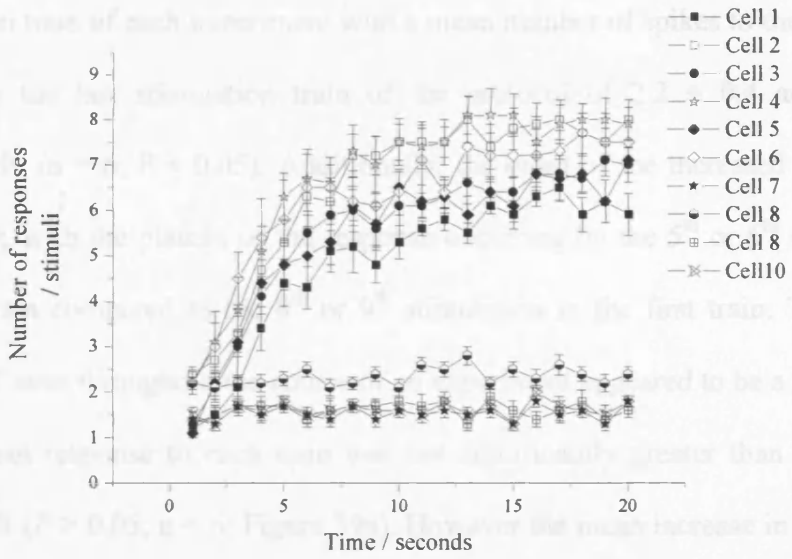


Figure 40 C-fibre response following stimulation of the face

Graph shows all cells responding with C-fibre latency following electrical stimulation of the face. Each data point represents the mean response to successive stimulations in a train of 20 stimuli, recorded over 10 trains. Cells 1-6 represent those cells that showed a significant increase in response or "wind-up" throughout the train ($P < 0.05$; $n = 6$). Cells 7-10 represent cells showing no significant increase in response throughout each stimulation train ($P > 0.05$; $n = 4$).

Interestingly, in the cells showing wind-up, the wind-up response in the last stimulation train in each experiment was significantly greater than in the first stimulation train of each experiment with a mean number of spikes to the first and last stimuli in the last stimulation train of the protocol of 2.2 ± 0.4 and 9.0 ± 0.7 respectively ($n = 6$; $P < 0.05$). Additionally, the onset of the increased response was also faster, with the plateau of the response occurring by the 5th or 6th stimulation in the last train compared to the 8th or 9th stimulation in the first train. The increased "wind-up" seen throughout the course of an experiment appeared to be a gradual effect as the mean response to each train was not significantly greater than the train that preceded it ($P > 0.05$; $n = 6$; Figure 39a). However the mean increase in wind-up over the first train became significant by the 6th stimulation train or 18 minutes after the start of the wind-up protocol ($P < 0.05$; $n = 6$).

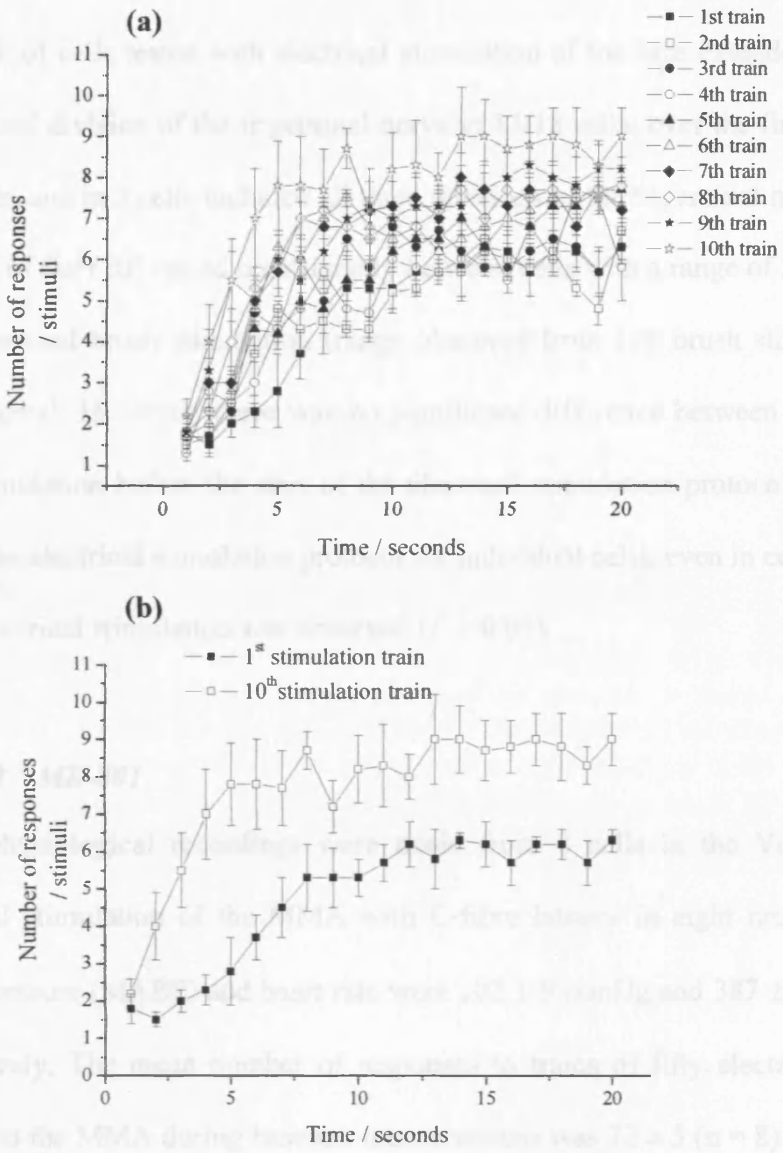


Figure 41 Wind-up increases during the experiment

Graphs show the mean response of cells showing wind-up following electrical stimulation of the face. (a) The mean wind-up response to each stimulation train gradually increases throughout the course of an experiment and by the 6th stimulation train wind-up is significantly greater than in the first stimulation train ($P < 0.05$; $n = 6$). (b) Mean wind-up in the ten stimulation train is significantly greater than in the first stimulation train ($P < 0.05$; $n = 6$).

5.3.2.3 Brush-pinch responses

The FRF of cells tested with electrical stimulation of the face extended over the first and second division of the trigeminal nerve in 13/18 cells, over the first division only in 2 cells, and in 3 cells included all three divisions of the trigeminal nerve. Responses to brush of the FRF varied considerably between cells with a range of 21 to 233 spikes per 10 second brush stimulation (range observed from 108 brush stimulations in 18 experiments). However, there was no significant difference between the response to FRF stimulation before the start of the electrical stimulation protocol and that at the end of the electrical stimulation protocol for individual cells, even in cells where wind-up of electrical stimulation was observed ($P > 0.05$).

5.3.3 MK-801

Electrophysiological recordings were made from 8 cells in the Vc responding to electrical stimulation of the MMA with C-fibre latency in eight rats. Mean arterial blood pressure (MABP) and heart rate were 102 ± 9 mmHg and 387 ± 6 beats/minute, respectively. The mean number of responses to trains of fifty electrical stimulation applied to the MMA during baseline measurements was 72 ± 5 (n = 8). Administration of MK-801 resulted in significant inhibition of the evoked response that was apparent in the first stimulation train after administration of 94 ± 6% below pre-treatment levels (n = 4; $P < 0.05$). Responses remained inhibited for the remainder of the experiment (45 minutes). MK-801 vehicle had no significant effect of MMA-evoked Vc responses at any stage during the experiment (n = 4; $P > 0.05$).

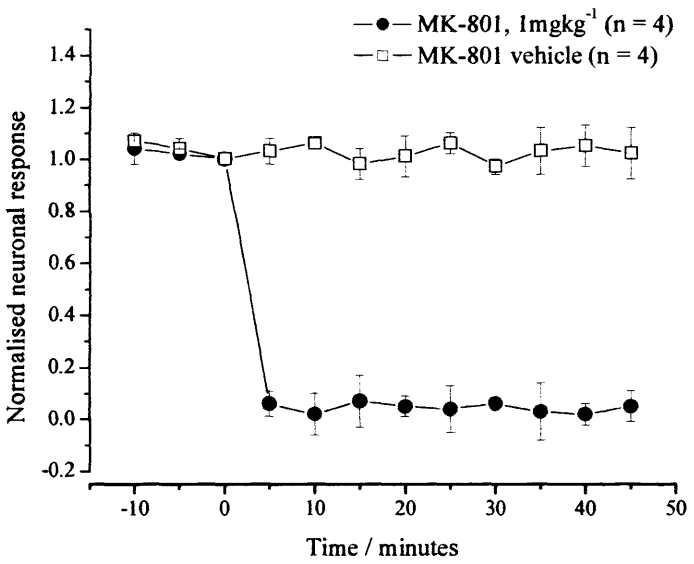


Figure 42 Effect of MK-801 on MMA-evoked Vc responses

Electrophysiological recordings were made as described in section 2.1-2.2. Data represents the mean normalised response of Vc neurons to trains of 50 electrical stimulations applied to the MMA. MK-801 (administered at time 0) resulted in significant inhibition of the evoked response that was apparent in the first stimulation train after administration of 94 ± 6% below pre-treatment levels ($n = 4$; $P < 0.05$). MK-801 vehicle (saline) had no significant effect of MMA-evoked Vc firing.

5.3.4 Mustard Oil (MO) stimulation

Electrophysiological recordings were made from 11 cells in the Vc responding to electrical stimulation of the MMA with Aδ-fibre latency in 11 rats. All had acceptable respiratory parameters for the anaesthetised rat: pCO_2, 35-45 mmHg; pO_2, 90+ mmHg and pH, 7.35-7.45. Mean arterial blood pressure (MABP) and heart rate were 115 ± 13 mmHg and 374 ± 10 beats per minute, respectively. Cells were located in the depth range 353-1296 μm below the pial surface. Average response latencies and dural stimulus threshold (current and pulse duration) were 13 ± 4 ms, 3 ± 0.5 mA and 0.3 ± 0.08 ms

In two experiments, MO application to the face, resulted in the loss of neuronal activity, hence, only data from six cells treated with MO have been included in the analysis. The mean number of responses to trains of fifty electrical stimulation applied to the MMA during baseline measurements of cells treated with MO was 69 ± 7 (n = 6). Following application of 10% MO to an area adjacent to the FRF, all 6 cells showed an increased firing rate above baseline. The mean maximum response occurred in the second stimulation train, 6 minutes after MO application where the mean number of responses to trains of fifty electrical stimulation was 103 ± 18 (49 ± 18% above pre-treatment firing, n = 6; $P < 0.05$). MO vehicle had no significant effect on response to electrical stimulation of the MMA and mean number of responses to trains of fifty electrical stimulation during baseline measurements and 10 minutes after MO vehicle application were 73 ± 4 and 76 ± 9 respectively (n = 3; $P > 0.05$).

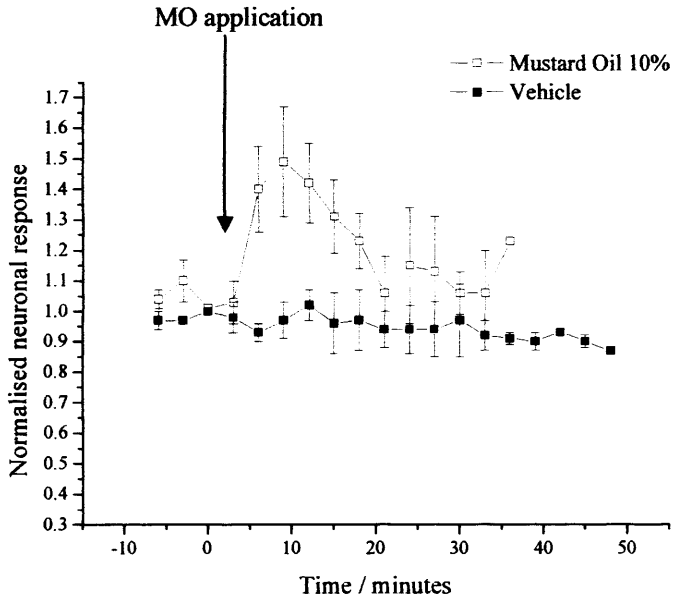


Figure 43 Effect of topical MO application on MMA-evoked Vc responses

Electrophysiological recordings were made as described in section 2.1-2.2. Data represents the mean normalised response of Vc neurons to trains of 50 electrical stimulations applied to the MMA. MO application (marked by the arrow) resulted in a significant facilitation of evoked response ($P < 0.05$, repeated measure ANOVA) compared to vehicle.(1 cell was "lost" 6 minutes after application).

The duration of the significantly increased response following MO treatment was variable between cells in a range of six to sixty minutes. Within this range, there was a trend for the cells showing the largest increase in firing to have the most prolonged effect as shown in the table below.

Maximum increase over baseline (%)	**Duration of effect / minutes**
110	60
79	18
66	18
23	13
48	6
33	6

Table 12 Duration of MO effect

The above table shows the maximum percentage increase over baseline recordings to trains of 50 electrical stimulations applied to the MMA following the application of MO to an area adjacent to the FRF and the time for which firing was significantly elevated over baseline levels for all 6 cells tested with MO.

Despite the presence of neuronal sensitisation, no cell tested with MO showed wind-up in any stimulation train throughout the experiment. The mean number of spikes to the first and last stimulus in the stimulation train before MO was 1.4 ± 0.4 and 1.4 ± 0.5 respectively and the mean number of spikes to the first and last stimulus in the second stimulation train after MO application was 2.0 ± 0.6 and 2.1 ± 0.5 respectively (n = 6).

All cells included in this study were classified as wide-dynamic range neurons based on their response to mechanical stimulation of the FRF. Two cells showed a FRF that was restricted to the first division of the trigeminal nerve, three cells showed a FRF that extended over both the first and second division of the trigeminal nerve and in one cell the FRF extended over the second and third division of the trigeminal nerve. An increase in the estimated size of the FRF determined by mapping with brush stimuli was observed in 4/6 cells exposed to MO at the end of the experiment compared to initial examination. No increase in the size of the FRF was observed in any of the cells tested with vehicle.

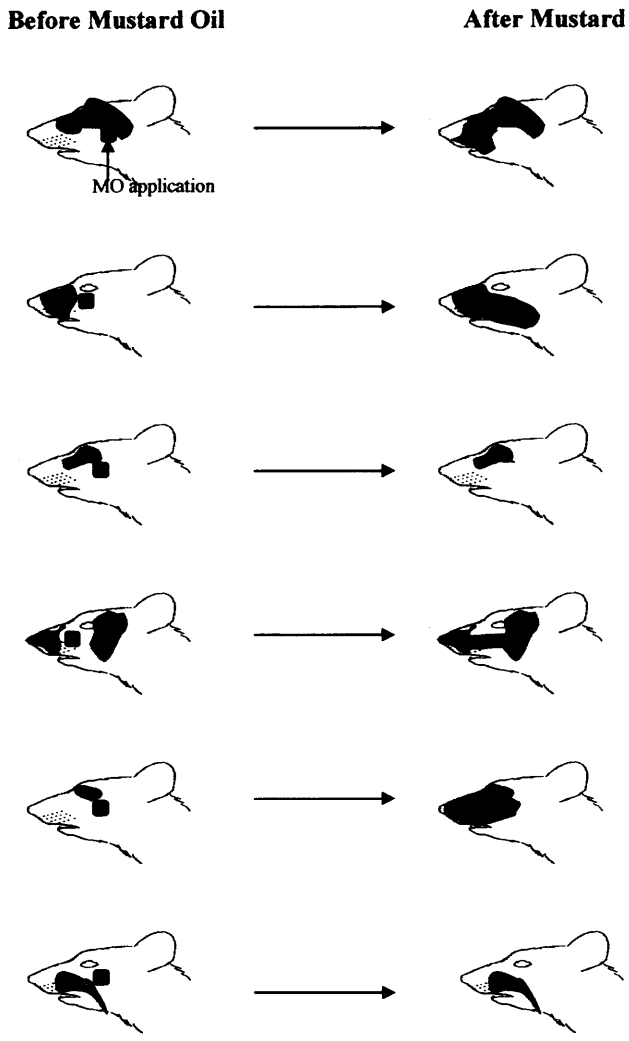


Figure 44 Expansion of FRF's following MO

Pictures represent the size of the FRF of Vc neurons, responding to brush stimulation, before MO application and at the end of the experiment.

MO also caused a significant increase in the response to light brush of the FRF compared to pre-treatment responses and vehicle treated animals. Due to the large variation in raw data between cells, pre-treatment responses were normalised to a value of one and recordings taken thirty and forty-five minutes after either MO or MO vehicle application are given as a normalised value in comparison to this. Control data represents nine observations at each time point from three experiments and MO data represents eighteen observations at each time point from six experiments. Thirty minutes after MO application, FRF responses were significantly elevated over those from animals treated with MO vehicle with a mean normalised response of 2.64 ± 0.21 (range; 1.7 - 4.8) and 1.27 ± 0.06 (range; 0.8 - 1.8) respectively in comparison to pre-treatment responses ($P < 0.05$). At the 45 minute time point, responses following MO were still significantly elevated over those from animals treated with MO vehicle, with a mean normalised response of 3.63 ± 0.38 (range; 1.6 - 6.7) and 1.36 ± 0.08 (range; 0.7 - 1.9) respectively in comparison to pre-treatment responses ($P < 0.05$).

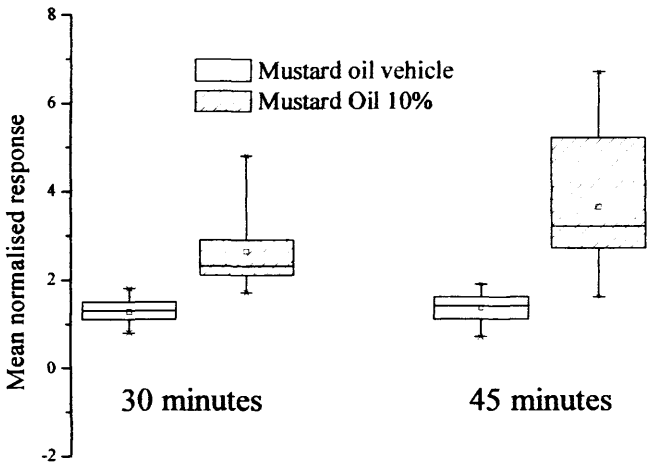


Figure 45 Response to FRF stimulation following MO application

Graph shows responses to a 10 second brush, 30 and 45 minutes after MO and vehicle application compared to pre-treatment responses, which have been normalised to a value of 1. Control data represents 9 observations at each time point from 3 experiments and Mustard oil data represents 18 observations at each time point from 6 experiments. Mean normalised response, thirty minutes after MO and MO vehicle application was 2.64 ± 0.21 (range; 1.7 - 4.8) and 1.27 ± 0.06 (range; 0.8 - 1.8) respectively in comparison to pre-treatment responses ($P < 0.05$). At the 45 minute time point, responses following MO were still significantly elevated over those from animals treated with MO vehicle, with a mean normalised response of 3.63 ± 0.38 (range; 1.6 - 6.7) and 1.36 ± 0.08 (range; 0.7 - 1.9) respectively in comparison to pre-treatment responses ($P < 0.05$).

5.4 Discussion

These experiments demonstrate that dural stimulation does not produce wind-up of second order Vc neurons. However, since this study demonstrates wind-up of neurons with facial stimulation, the methods employed are sensitive enough to detect the phenomenon. Moreover, this study also demonstrates that the process of wind-up following activation of C-fibres during electrical stimulation of the face, leads to a persistent increased neuronal hyperexcitability evidenced by the increase in wind-up throughout the course of an experiment. As wind-up of C-fibre responses can be demonstrated in the Vc following FRF stimulation and have previously been shown following tooth pulp stimulation (Hamba *et al.*, 1992), the lack of wind-up in Vc neurons to dural stimulation is not a general characteristic of the trigeminal system. Rather, it is likely that the differential response following stimulation of dural and facial afferents relates to a difference in the processing of visceral and cutaneous inputs. This difference may be attributed to different classes of sensory receptors and pre-synaptic mechanisms, distinct populations of second order neurons with diverse post-synaptic mechanisms or central inhibitory mechanisms.

A major limitation of this study is the absence of recording from a convergent neuron responding to electrical stimulation of both the face and dura mater, which would allow categorical determination of whether the Vc neuronal population exhibiting wind-up in response to facial stimulation is the same population that do not wind-up following dural stimulation. However, the neuronal population responding to dural stimulation examined in this study also exhibited a mechanical FRF. As mechanical stimulation of the face was used to identify neurons for the electrical facial stimulation

protocol and it was very rare it find a cell responding to mechanical stimulation but not electrical facial stimulation, it is likely that the population of Vc neurons extends over the same pool. However, an alternating electrical stimulation protocol between dural and facial afferents that activated a convergent Vc neuron would lend weight to the suggestion that the neuronal population is the same. Additionally, it would be of interest to observe whether there was sensitisation of the dural response following a wind-up response after facial stimulation.

Wind-up or temporal summation of afferent input is a central not peripheral phenomenon. In this study, the majority of neurons receiving a C-fibres input were found in the deeper layers of Vc. However, direct connections between nociceptive neurons and C-fibres are rarely found here, with the majority of C-fibres terminating in the superficial layers (Ambalvanar and Morris, 1992; Brown, 1981; Hunt *et al.*, 1987) and reaching the deeper layers via interneurones located in the substantia gelatinosa. Hence, amplification of C-fibre input may occur either in the superficial or deeper layers and as such the difference between the response to dural and facial stimulation may result from differential interneuron connections.

WDR neurons in the trigeminal nucleus oralis (Vo) have been found to resemble those in lamina V of the spinal dorsal horn in their response mechanical, thermal, chemical and electrical cutaneous stimuli as well as to non-noxious stimuli (Raboisson *et al.*, 1995). Activation of C-fibers in the Vo evoked by high intensity stimulation of their receptive field have also been shown to exhibit windup during low frequency (0.66 Hz) suprathreshold electrical stimulation with a dependence on NMDA receptors (Mendell, 1966; Wagman & Price, 1969; Urban & Randic, 1984; Woolf *et al.*, 1988;

Yoshimura & Jessell, 1989; Thompson *et al.*, 1994; Woda *et al.*, 2004). Features common to the Vo and lamina V of the spinal dorsal horn is that cutaneous C-fibers terminate in the superficial laminae of the dorsal horn only and do not contact directly WDR neurons located either in lamina V of the dorsal horn or in Vo (Light & Perl, 1979a; Light & Perl, 1979b; Ralston, III & Ralston, 1982; Sugiura *et al.*, 1986; Ambalavanar & Morris, 1992). Opioid receptors (Atweh & Kuhar, 1977; Arvidsson *et al.*, 1995; Ding *et al.*, 1996) as well as substance P (Sugimoto *et al.*, 1997) are not seen in significant amount within these regions. Additionally, morphine has no direct effect upon lamina V Vo or dorsal horn WDR neuron activity but can inhibit their response to noxious stimuli when injected in the corresponding superficial laminae of the dorsal horn (Duggan *et al.*, 1977; Fleetwood-Walker *et al.*, 1988; Dallel *et al.*, 1998).

This evidence has led to the speculation that interneurons that transmit the nociceptive input from the superficial laminae provide the glutamatergic input to deep dorsal horn and Vo WDR nociceptive neurons and windup depends upon NMDA receptor activation at the end of the pathway which allows WDR neuron activation by C-fibers (Woda *et al.,* 2004). This would be in keeping with results presented in this thesis, where a dependence on NMDA receptors of MMA-evoked responses is demonstrated by the inhibition of responses following administration of an NMDA antagonist. As no wind-up is observed in the Vc following MMA stimulation it would suggest a modulation of afferent input before activation of the NMDA receptor.

Studies examining wind-up in the Vc are sparse but there are reports of wind-up following tooth-pulp stimulation (Hamba *et al.*, 1992) and of the FRF (Luccarini *et al.*, 2001). Interestingly, NMDA intrathecally applied at the level of the medulla was found to induce differential effects, either excitatory or inhibitory, on convergent (wide dynamic range) neurons of the Vc according to their location in the superficial or deeper laminae, with an increase in the evoked activity of superficial neurons and decrease in the activity of deeper neurons (Luccarini *et al.*, 2001). This is in contrast to the spinal cord where NMDA has generally been reported to have an excitatory effect on the nociceptive activity of convergent neurons (Chapman *et al.*, 1994; Sher *et al.*, 1994; Zhang, 1996, 1998), although there is some evidence that an inhibition may also be the final result of NMDA receptor activation (Dickenson & Sullivan, 1990; Svendsen *et al.*, 1999).

The authors of this study proposed that the inhibitory effect of NMDA on the activity of deep neurons is not likely explained by a direct action of the agonist at the level of the neurons since the micro-iontophoretic application of NMDA on deep neurons always induces excitatory responses (Zhang, 1996). It is possible that the NMDA-sensitive superficial neurons act as inhibitory interneurons on deeper nociceptive neurons. Other differences also exist between convergent neurons in the superficial and deep laminae of Vc and deep convergent neurons typically exhibited consistent wind-up contrary to the mild wind-up of more superficial neurons (Dickenson & Sullivan, 1990). As Vc convergent neurons in the superficial laminae of the Vc are known to be contacted by enkephalinergic, noradrenergic and serotoninergic inhibitory pathways originating from supraspinal centers (Sessle, 2000). It is possible that

facilitated superficial neurons were part of a local inhibitory circuit, which when activated trigger descending influences from a supraspinal loop resulting in neuronal inhibition in the deep laminae.

Also of interest in the study examining wind-up following electrical stimulation of the face is the time taken to reach the maximum level of wind-up or "plateau phase", which in our study, decreased throughout the course of an experiment. This plateau phase can also be observed in dorsal horn neurons and both a supraspinal mediated inhibition (Gozariu *et al.*, 1997) and a post-tetanic depression of neuronal firing (Woolf, 1983) have been proposed to counteract the increase in response that occurs throughout the electrical stimulation train. Interestingly, C-fibre evoked potentials recorded in the superficial dorsal horn are reported to continue showing a wind-up response through trains of up to 100 stimuli (Schouenborg, 1984). Given the apparent difference in modulation between wind-up of dorsal horn neurons and C-fibre evoked potential, we cannot rule out the possibility that neurons activated by visceral inputs at intensities sufficient to generate wind-up, activate powerful inhibitory pathways preventing the manifestation of wind-up. The triggering of inhibitory modulation by noxious stimulation is a well established process (Bouhassira *et al.*, 1995; Fields & Basbaum 1993), which is demonstrated by loss of the plateau phase normally seen during wind-up of dorsal horn neurons following spinalisation (Gozariu *et al.*, 1997). Activation of descending inhibitory systems, triggered by spatial summation of nociceptive inputs has been shown to be highly dependent on the integrity of areas situated in the most caudal part of the medulla in a negative feedback loop that is independent of the more rostral brainstem structures (Gall *et al.*, 1998). It is possible that activation of dural afferents in a temporal manner may cause activation of a

similar inhibitory system. The involvement of brainstem structures such as the PAG, LC or hypothalamus in modulating wind-up of Vc neurons following tooth pulp stimulation has previously been demonstrated by a partial blockade of the response after conditioning stimuli applied to the arcuate nucleus of the hypothalamus (Hamba *et al.*, 1992). However, their role in modulating the response to stimulation of the dural receptive field remains to be investigated but the importance of understanding where the deviation in visceral and cutaneous pathways occurs warrants further investigation of these inhibitory mechanisms.

This study also demonstrates that the process of wind-up following activation of C-fibres during electrical stimulation of the face, leads to increased neuronal hyperexcitability evidenced by the increase in wind-up throughout the course of an experiment. It is however, interesting that at the end of the electrical stimulation protocols, in cells showing wind-up, no enhancement of the response to mechanical stimulation of the FRF was seen. This observation is in keeping with current thinking that wind-up is able to generate some of the characteristics of central sensitisation but is not sufficient to model all of the central changes that might occur following peripheral injury (Herrero *et al.*, 2000).

The experimental stimulation of dural afferents, as with many visceral structures, also adds a degree of complexity to the interpretation of the data, as surgery is normally required for access, hence, tissue sensitisation is likely. Regarding the study of dural and cerebral blood vessels and their central terminals, both closed cranial windows, where the skull is thinned and open cranial windows, where the bone is removed over the area of interest, have been previously used in experimental animal models

(Cumberbatch *et al.,* 1998, 1999; Burstein *et al.,* 1998; Strassman *et al.,* 1996). In preparations where an open cranial window has been used, the reported stimulus intensities required to activate both Aδ and C-fibres are in the range; 0.8ms, 0.5-4mA and 1Hz (Burstein *et al.,* 1998). However, in our preparation with a closed cranial window, these parameters would typically only elicit an Aδ-fibre mediated response, with C-fibre activation typically requiring at least double that current amplitude and pulse width. This difference in stimulation parameters between the studies may be due to dissipation of the stimulus with a closed cranial window but may also indicate a greater presence of peripheral sensitisation following a craniotomy. Nevertheless, in either preparation, some degree of sensitisation is likely, consequently, we are only able to speculate whether dural afferents are normally "silent" and become responsive during times of inflammation or whether they fall into a class of nociceptive-specific sensory receptors.

The character of Vc neurons following the induction of central sensitisation with MO was also explored here. In agreement with data from other studies, MO resulted in a sensitisation of evoked Vc neuronal responses (Bartsch & Goadsby, 2002; Hu *et al.*, 1992). Sensitisation typically lasted between 6 and 18 minutes, however in one cell, which also showed the largest response, sensitisation remained for the duration of the experiment (60 minutes). Interestingly this cell was found in the superficial layers of Vc, where as all other recordings were from the deeper layers of Vc. As MO was applied outside the FRF in these experiments, the observed facilitation of both noxious electrically and innocuous mechanically evoked responses are likely to be the result of central changes reflective of the process of heterosynaptic facilitation, which is further supported by the observed expansion of the FRF. Interestingly as with PGE_2, the

sensitisation of mechanical responses following MO application outlasted the sensitisation of electrically evoked responses, further emphasising that while a peripheral input is necessary to initiate central changes, the maintenance is independent of an ongoing peripheral input.

In summary, the manifestation of neuronal wind-up is likely to be a function of activation of differing neuronal pathways dependant on primary afferent input. As such, some forms of pain serve to cause reflex withdrawal from the nociceptive stimuli with ongoing pain and inflammation ensuring the affected area is rested to allow recovery from injury, such as may be the case with nociceptive inputs from skin, mucosa and teeth. However, migraine head pain seems unlikely to fulfil this type of protective role and is more akin to deep visceral pain. Indeed, theories of migraine are in good agreement with the pain having a visceral origin, indeed a recognised stimulus of visceral pain is ischaemia and it is postulated that pain may originate from the accumulation of inflammatory mediators in the ischaemic tissue (Malliani, 1986; Ness & Gebhart, 1990). A reduced blood flow in dural and cerebral afferents have long been suggested to occur during migraine attacks (Blau, 1978; Caekebeke *et al.,* 1992; Diener & Limmroth, 1993; Shevel & Spierings, 2004) and the resultant build up of inflammatory mediators may underlie the activation of dural afferents. Indeed, the introduction of inflammatory mediators into to the dural receptive field is associated with an enhanced response in the Vc to both mechanical and electrical stimulation of the dura (Burstein *et al.,* 1998). This study has demonstrated that wind-up of dural afferent input is not likely to contribute to central sensitisation in conditions of dural activation. Given the extensive data that has been generated on pain pathways originating from cutaneous sources in recent years and the relative lack of

understanding of visceral pain pathways, understanding why wind-up does not occur following stimulation of dural afferents compared to facial stimulation may further the understanding of how central sensitisation is induced in conditions such as migraine.

6 General Discussion

The results in this thesis support the hypothesis that a sensitisation of VBNC neurons generates an altered neuronal activity that may underlie some of the clinical features of migraine such as head pain and facial skin hypersensitivity. Furthermore, these studies also demonstrate that this central sensitisation can occur following introduction of inflammatory mediators into the peripheral dural receptive field. Hence, a crucial question that needs to be addressed is the physiological relevance to understanding migraine of the studies described.

What is the physiological relevance of these models to migraine?

One of the first issues that needs to be addressed regarding the studies described here is the validity of the models used. The main method used in these studies was *in vivo* electrophysiology. The advantage of this technique is that is allows the study of a single cell within the CNS in a physiological setting where the cell is under the influence of both supraspinal and local modulatory circuits. However there are also distinct disadvantages of this type of *in vivo* study, such as the effects of anaesthesia, as well as various unknown factors, for example the physiological relevance of electrical stimulation of the MMA. As discussed earlier electrical stimulation of the dura mater is almost certainly nociceptive in character. This is supported by many factors, such as the lack of innervation of the dura mater by A-β fibres, the elicitation of A-δ and C-fibre response following electrical stimulation. Additionally NMDA antagonists potently inhibit MMA evoked Vc activity (see section 5.3.3.) suggesting the involvement of the NMDA receptor in Vc evoked firing which is widely accepted

only to be involved in nociceptive signalling. Hence, how is the modulation of an already nociceptive signal by inflammatory mediators related to nociceptive processing in migraine headache?

Under physiological conditions, it is likely that the primary afferents innervating the dura mater are largely silent. During the pathophysiological processing occurring in migraine however a variety of factors could result in the systemic activation of the primary afferent fibres. In the first case, abnormal distension of intracranial vessels may result in mechanical activation, secondly, dural inflammation may result in the release of mediators directly capable of chemical stimulation, and either of these two processes may then stimulate the other to occur in a positive feedback cycle. Other possible mechanisms of trigeminal activation include activation by the efferent terminals of the autonomic nervous system or antidromic activation from the ophthalmic division of the trigeminal nerve. While it is not known if any or all of these factors actually occur to manifest as the throbbing head pain experienced during migraine, electrical stimulation of the MMA, which directly activates the nerve, is likely to be representative of the effects of all of the above situations. Hence, until more is known about the processes leading to dural activation, there is a good rationale for using MMA stimulation in preliminary models of migraine pain.

However there are several caveats of MMA stimulation, the most obvious being the visualisation of the vessel by thinning the overlying skull. The mechanical disruption occurring to the underlying tissue during drilling is undoubtedly going to generate some inflammation even in the hands of the most experienced surgeon. This is supported by the intravital microscopy studies, where once visualised through a

microscope, the MMA was amplified onto a large TV screen and during the 15-20 minutes after drilling, a significant vessel contraction could be observed. For this reason, experimental protocols were never started until at least 60 minutes after the completion of drilling. The other issue with electrical stimulation of the MMA for model purposes is the trains of stimuli used. The main stimulation parameters in these studies involved either trains of 20 stimuli applied every 3 or 50 stimuli applied every 5 minutes. While this pattern of activation is unlikely to occur physiologically it obviously necessary to have defined periods of data collection that are statistically meaningful, allowing both comparison of mean response between stimulation trains and within stimulation trains. Furthermore, during the characterisation of this model, neuronal responses were found to be reproducible for 3 to 4 hours in response to either stimulation protocol. Even after this time point, the loss of reproducibility was largely due to drift of the electrode, such that neuronal responses fell below the discriminator window, rather than an alteration in the number of responses seen to each stimulation train. Hence, it is unlikely that electrical stimulation of the dura mater fundamentally altered neuronal characteristics of the cell under study; as such, the use of MMA stimulation is a good measure of trigeminal nociceptive signalling albeit in an un-physiological pattern of activation.

The use of *c-fos* expression following peripheral activation of A-δ and C-fibres also has limitations. Within the VBNC, the full range of receptors able to generate the induction of *c-fos* is not yet known, however several receptor agonist / antagonists can attenuate *c-fos* expression (e.g. NK-1 antagonist, 5-$HT_{1B/1D}$ agonists, $GABA_A$ agonists) but not all of these compounds have anti-migraine like activity (for review see Mitsikostas & del Rio, 2001). In addition, *c-fos* expression represents slow nuclear

changes, with mRNA alterations thought to be maximal approximately 2 hours after the stimulus and alterations in protein expression approximately 4 hours after the stimulus, hence it is unlikely to be involved in acute responses to peripheral nociceptive signals. The study of VBNC *c-fos* expression is a useful measure of the location of activated cells following trigeminal nociceptive firing but caution should be used in interrupting these data. It is possible to speculate that the induction and degree of *c-fos* induced may be an indicator of plastic changes occurring in the VBNC over prolonged periods of time. For example, the downstream processing of *c-fos* activation may modulate potential slow transcriptional changes responsible for receptor up-regulation, however, these possibilities currently remain unexplored.

A further question regarding the physiological relevance of these data concerns the species and strain used. While rats are frequently used to model many diseases because of their similar anatomy to the human, their relative ease of breeding, their tolerance to long periods of anaesthesia and the wealth of literature available about experimental procedures on them, there are also many complicating factors. With particular relevance to migraine, the 5-HT receptor pharmacology appears to be considerably different from the human and for this reason; much of the experimental work supporting development of the triptans was carried out on guinea pig tissue. However, in our laboratory, considerable problems were found in anaesthetising guinea pigs, hence for studies such as *in vivo* electrophysiology, which can involve periods of anaesthesia from 3 to 10 hours, rats are a reasonable species choice.

Despite awareness and the caution used in interpreting data from different species, there is less conclusive data concerning strain differences within species. The majority

of studies in this thesis and all *in vivo* electrophysiology experiments used Sprague Dawley rats (supplied by Charles River). However a comparison of the effects of a full adenosine A_1 agonist and partial A_1 agonist has also been carried out in the same *in vivo* electrophysiology preparation using both Sprague-Dawley rats and Wistar rats, supplied by Bantin and Kingman laboratories (Bolton *et al.* unpublished data). In these studies, the full agonist significantly inhibited MMA evoked neuronal activity in both strains of rat, however the partial agonist inhibited MMA evoked neuronal activity in the Wistar rats only, with no effect on the evoked activity in Sprague dawley rats. While the mechanism of this different effect is only the subject of speculation and could either be specifically related to the involvement of the adenosine A_1 receptor in trigeminal firing or a non specific pharmacokinetic effect, it does highlight considerable strain differences exist.

Strain differences have also been described by others for example, Benoliel and colleagues showed that following chronic constriction of the infraorbital trigeminal nerve, Lewis rats developed considerable hyperalgesia following a secondary insult that did not occur in Sprague Dawley rats (Benoliel *et al.*, 2002),. However, following nerve ligation in the lumbar region, Sprague Dawley rats and Lewis rats showed very similar pain and hyperalgesia responses (Yoon *et al.*, 1999). While current available data does not allow generalisation about which particular strain of rat is most suitable for a particular model, it does suggest that future work should give the issue more consideration before embarking on development of a particular model.

Do these studies provide evidence for a peripheral "generator" of migraine?

The issue of a peripheral versus a central "migraine generator" is the oldest and still the most controversial source of debate among migraine researchers. Many believe that certain migraine "triggers" are sufficient to initiate peripheral inflammatory processes in susceptible individuals. The consequence of peripheral activation then resulting in a sequential time order dependent generation of sensitisation in second and third order neurons in the VBNC and brainstem respectively. If this is the case, then other questions need to be addressed such as the presence of features that are likely to have a central origin, for example, premonitory symptoms, the episodic nature of the disease, nausea and sensitivity to light and sound.

It is possible that the symptoms of migraine that are of central origin are reactive responses to sequential sensitisation of third order neurons. However while this may hold true for features such as nausea, photophobia and phonophobia, it would not account for the premonitory phase, which can start up to 48 hours before the headache. Hence, if a peripheral sensitisation were the trigger event for a migraine attack, the process would also have to start several days before the headache. This would raise some interesting questions, such as how would some of the trigger factors of migraine such as certain foods, stress, alteration in daily routine, initiate this process and what intracellular processes could account for such a long duration of action in the periphery?

The effect of factors such as stress that trigger migraine in susceptible individuals is extremely complex and not easily studied as the physiological response varies between

individuals and is not fully understood. However, considerable efforts have been made to understand how and where the effects of substances, such as GTN, that can act as a trigger, occur. While the effects of GTN in generating migraine are delayed by 4 or 5 hours following intravenous infusion, the time scale is not in agreement with a peripheral response generated over several days. Furthermore, GTN is likely to have widespread effects in both the peripheral and central nervous system, hence even if activation of a peripheral mechanism could be observed following GTN infusion, a central basis for the effects could not be excluded.

The effects of PGE_2 on the response of second order neurons in the VBNC described in study 2 of this thesis, provide the greatest evidence that peripheral sensitisation of primary afferents innervating the dura mater can generate a neuronal effect that correlates well with the clinical features of migraine. However, the induction of endogenous PG synthesis is likely to occur only under conditions of inflammation, which could equally occur in response to a peripheral or central trigger.

If headache is on a sliding scale such that the underlying pathology of tension type headache and migraine is similar but of greater intensity in migraineurs, it is conceivable that trigger factors resulting in an activation of intracranial structures generate a meningeal inflammation that is also of graded intensity. Alternatively, the tendency of certain individuals to suffer recurrent migraineous episodes, may result from a greater genetically determined susceptibility of dural afferents to activation. However, neither scenario seems likely as they would not explain features such as the predominant unilateral localisation of migraine pain compared to the bilateral localisation of the head pain in tension type headache sufferers. Hence, while these

studies support the role of a peripheral inflammation in the development of migraine headache, evidence currently available suggests that the effect is secondary to a central dysfunction.

Do these studies provide evidence for a central "generator" of migraine?

In support of a central dysfunction as the factor generating susceptibility to migraineous episodes is the data from the MO studies. In these studies application of the C-fibre activator outside the receptive field of the cell under study resulted in a significant facilitation of the response to MMA stimulation. This demonstrates that a peripheral sensitisation of cutaneous trigeminal afferents can result in central changes that are propagated throughout the VBNC causing a widespread hyperexcitability. Hence, it is also likely that excitatory input to the VBNC from other areas may result in a similar generalised increase in excitability. While this alone does not a indicate that a facilitation of excitatory descending pathways or an inhibition of descending inhibitory pathways generates a sensitisation of the VBNC, it does indicate that this type of effect would be sufficient to render the VBNC hypersensitive.

A dysfunction of supraspinal controls has previously been discussed in chapter 1 and may well result from genetic mutations manifesting as defective calcium channels present in structures such as the PAG or hypothalamus. However, it is also possible that no distinct "dysfunction" exists and the balance of descending inhibitory and excitatory controls is related to a threshold effect that varies between individuals. In either case normal activation of trigeminal afferents would be likely to result in a similar exaggerated response seen after peripheral sensitisation.

It should also be remembered that while these studies have focused on abnormal activity of the trigeminal system, it is also possible that the pathology of migraine lies outside of the trigeminal system. The much greater incidence of migraine in females strongly suggests that the target organs of female sex hormones may play a crucial role in determining migraine susceptibility. Hence while current research does not adequately answer the "central versus peripheral debate", research directed towards understanding how only some individuals develop a tendency to suffer recurrent attacks may well point to the elusive "migraine generator".

Future work

Several areas where considerable improvements could be made to current preclinical migraine models are:

- Further characterisation of Vc electrophysiology: The studies described in this thesis provide a preliminary report on the effect of various inflammatory mediators on electrophysiological recordings of second-order Vc neurons, however the list is by no means exhaustive and the effect of many other transmitters and peptides on trigeminal nociceptive activity, remain un-investigated. Of most interest for future investigation, is the elucidation of receptor mediated responses to substances that are likely to be produced endogenously during inflammation such as the PGs. Further value would also be added to these experiments by the sampling and analysis of blood neuropeptide content at various time points throughout a protocol.

  Recently there has been a surge in interest in the role of spinal PGs in generating

hyperalgesia and allodynia. There are a multitude of studies examining the involvement of PGs in centrally mediated responses following both experimental induction of peripheral sensitisation and direct application of PGs to the spinal cord (see table below for a summary of some of these findings). Experimentally, the induction of allodynia seems to be dependant on activation of EP_1 receptors and occurs over a time course of 5-50 minutes after application PGE_2. Interestingly PGE_2 induced allodynia appears to be modulated by glutamate receptor antagonists, morphine and NOS inhibitors (Taiwo & Levine 1988).

The characteristics of experimental hyperalgesia induced by intrathecal PGE_2 are similar to those of allodynia with reliance on glutamate and NO for a functional response. However, knock out mouse studies have indicated that the hyperalgesic response is likely to be mediated through the EP_2 and EP_3 receptors (Minami *et al.*, 2001) rather than the EP_1 receptor. The EP_2 and EP_3 receptors have also been identified by Kumazawa *et al.*, (1996) as mediating peripheral enhanced responses to bradykinin. These observations led to the suggestion that EP_2 and EP_3 mRNA, present in DRG's, may be transported to the periphery following peripheral insult, hence mediating both a central and peripheral effect of PGs (Ito *et al.*, 2001). Hence it would also be of interest to look at the role of central PGs in trigeminal processing.

In Vivo Experiments				
Ligand	**Test**	**Dose**	**Effect**	**References**
PGE_2 (EP_3> EP_4> EP_2> EP_1>)	Paw pressure	2.5-10ng/rat	hyperalgesia 4 + hours	Ferreira & Lorenzetti, 1996
	Paw stroking	50pg-5μg kg^{-1} (it)	allodynia 5-60minutes	Minami *et al.*, 1996 Minami *et al.*, 1994
	Hot plate	1pg-500ngkg^{-1} (it)	hyperalgesia 15-60minutes	Nishihara *et al.*, 1995 Taiwo & Levine 1998
	EAA & GABA release	2-7μg/rat (it)	increase	Jurna *et al.*, 1993
17-phenyl-trinor-PGE_2 (EP_1 ~EP_3)	Vc electrophysiology	1-10nmol (icv)	hypoalgesia	Oka *et al.*, 1997
	Hot plate	500pg into VHM	hypoalgesia	Hosoi *et al.*, 1999
	Hot plate	5pg-5ngkg^{-1} (it)	hyperalgesia	Minami *et al.*, 1994
Butaprost (EP_2)	Hot plate	50ng-5μgkg^{-1} (it)	allodynia	Minami *et al.*, 1994
MB28767 (EP_3)	Vc electrophysiology	1-100fmol (icv)	hyperalgesia	Oka *et al.*, 1997
GR63799 (EP_3)	Tail flick	20-300ng (icv)	hyperalgesia and fever	Xin *et al.*, 2000
Sulprostone (EP_3>EP_1)	Hot plate	50pg-5ugkg^{-1} (it)	allodynia	Minami *et al.*, 1994

In Vitro Experiments					
Ligand	**Test**		**Concentration**	**Effect**	**References**
PGE_2	Superficial dorsal horn neurone -evoked EPSCs -mEPSCs		1μM	facilitation 10-40 mins	Minami *et al.*, 1999
	DRG	TTX-R I_{Na}	1μM	increase	Gold *et al.*, 1998
		-capsaicin induced - I_m	0.1-1μM	increase ~ 15 minutes	Lopshire & Nicol 1997
		BK-induced $[Ca^{2+}]_{IC}$	100nM	facilitation	Stucky *et al.*, 1996
		firing	1μM	increase	Cui & Nicol 1995
		CGRP release	0.1-1μM	increase, 2-5 minutes	Hingtgen *et al.*, 1995
	TG spontaneous firing		0.1μM	increase	Baccaglini & Hogan 1983

Table 13 Central action of prostaglandins

Summary of some of the experimental findings using prostaglandins and their synthetic ligands in pain models. Adapted from Vanegas & Schaible (2001). (abbreviations: it- intrathecal; VHM-ventromedial hypothalamus; icv- Intracerebroventricular injection; EAA-excitatory amino acids; TG-trigeminal ganglion; EPSC- excitatory post-synaptic current).

- Development of recovery animal models: One of the major issues with current animal models is the generation of sensitisation *per se.*, following surgery. Chronic implantation of stimulating devices or cannula, through which substances could be administered, would allow the testing of substances after surgical inflammation had subsided. Additionally it would also allow assessment of behavioural effects of stimulation which could later be assessed at the single cell level in anaesthetised preparations.

- Development of behavioural testing: While we can speculate that neuronal activity in the Vc is representative of pain experienced during a migraine, a behavioural read out that could be used in parallel with these type of experiments would be extremely useful. The features of migraine that could potentially be best utilised for behavioural testing are the sensitivity to light and sound. While it may also be possible to measure pain thresholds on facial skin in the same way as paw withdrawal thresholds are often assessed in neuropathic pain models, this would be complicated by the vibrissal innervation as even innocuous activation of a single vibrissae results in a withdrawal reflex.

Also of interest would be the use of *in vitro* techniques to develop migraine models. In particular, the use of molecular biological assays such as PCR to investigate the

phenotypes of trigeminal primary afferents innervating dura mater and cutaneous tissue, may provide significant advances in understanding the differential effects of cutaneous and visceral stimulation. *In vitro* electrophysiological recordings would also be useful in examining the behaviour of Vc neurons in the absence of descending control systems, which to some extent has been assessed by recordings on slices of Vc. Further neuronal characterisation could further be obtained by looking at cultured neurons, so that local neuronal networks were also disturbed. This was explored in the developmental stages of this thesis and while at the time, investigation of the *in vivo* situation was considered more pressing, the generation of *in vivo* data over the last couple of years would now make the comparison with *in vitro* data extremely interesting. Additionally, use of tissue taken from *in vivo* migraine studies would further the understanding of molecular changes responsible for some of the effects seen *in vivo.*

Summary

The two main theories of migraine headache involve either a dysfunction of the cranial vessels and their trigeminal innervation or a central neural generation of symptoms. In either situation, sensitisation of VBNC neurons is a possible consequence. The studies described in this thesis show that sensitisation of VBNC neurons can be elicited by peripheral sensitisation of the dural receptive field and a cutaneous area innervated by the trigeminal nerve, that is in keeping with the time scale over which allodynia and hyperalgesia develop clinically. Further investigation of how the process of sensitisation is initiated is likely to provide breakthroughs in the understanding of migraine pathophysiology.

7 Appendix

7.1 Appendix A-Drugs and dilutions

All doses refer to free base weight.

7.1.1 Anaesthetics

Alpha-chloralose: Alpha-Chloralose was obtained from Sigma (Sigma-Aldrich company, Dorset, England) (C-8091) and prepared by mixing with an equal weight of Borax (Sigma). It was then diluted in saline to a concentration of 80mg/ml (dose=1.25ml/kg), warmed to 40 ^{o}C and stirred continually using a magnetic stirrer.

Euthanal: Euthanal (sodium pentobarbitone) was obtained from the distributor Genusxpress (Lancashire, UK) and manufactured by Merial (UK). It was used at the end of experiment for euthanasia and 0.5ml given iv, undiluted from stock (200mg ml^{-1})

Isoflurane: Isoflurane was obtained from the distributor Genusxpress (Lancashire, UK) and manufactured by Merial (UK). For the induction of anaesthesia, animals were exposed to 5% in oxygen. For maintenance of anaesthesia, 3% in oxygen was used.

Sagittal: Sagittal (sodium pentobarbitone) was obtained from the distributor Genusxpress (Lancashire, UK) and manufactured by Rhone Merieux (UK). After discontinuation of isoflurane anaesthesia, 1ml of a 20mgml^{-1} solution was administered iv. Thereafter, a constant infusion of between 10 and 30mgkg^{-1}hr^{-1} was given.

Urethane: Urethane was obtained from. Doses of up to $5gkg^{-1}$ ip were given. Due to adverse effects, the use of Urethane was governed by a risk assessment.

Vecuronium bromide: Vecuronium bromide was obtained as Norcuron from Sigma. Dilutions were made in saline.

7.1.2 Study 1 test compounds

Capsaicin: Capsaicin was obtained from Sigma. It was dissolved in 10% tween 80, 10% ethanol and 80% saline.

CGRP: Rat-αCGRP (Bachem, UK) was initially dissolved in distilled water and aliquots were frozen, subsequent dilutions were made in 0.9% saline.

GTN, intra-arterial studies: Glyceryl trinitrate was obtained from Merck Pharmaceuticals as the commercially available product Nitronal and was infused undiluted from stock solution ($1mgml^{-1}$).

GTN, intra-venous studies: GTN was obtained as a 1mg/ml solution in a non-light sensitive formulation (Schwarz Pharma Ltd, UK) and diluted in glucose saline.

LPS: LPS (Lipopolysaccharide from E.coli, serotype 0111:B4) was obtained from Sigma. It was diluted in saline and frozen as a $4mgml^{-1}$ solution. Subsequent dilutions (20μg in 50μl) was also made in saline.

NGF: NGF (Nerve growth factor) was obtained from Sigma. Dilutions were made in saline and administered as 2.5μg in 50μl

Sumatriptan: Sumatriptan (SKF-98515-K) was donated by GSK as the hydrogen succinate salt and diluted to a dose of 300μg/kg in 0.2ml.

7.1.3 Immunohistochemical reagents

Rabbit serum: Rabbit serum was obtained from Vector Laboratories (Peterborough, UK) and diluted in 98% immunobuffer.

Normal Goat Serum: Normal goat serum was obtained from Vector Laboratories (UK). 10% normal goat serum (NGS) was used for incubation and diluted in PBS + 0.2% triton.

Primary *fos* antibody: Primary fos antibody was obtained from Sigma Genosys. (Sheep anti-fos OA-11-824) and reconstituted with distilled water. Dilutions were made in1μl rabbit polyclonial antisera, 1ml NGS and 19ml PBS containing 0.2% triton to give a 1:20,000 dilution.

Secondary biotinylated rabbit anti-sheep antibody: Secondary biotinylated rabbit anti-sheep antibody was obtained from Vector Laboratories (UK) and diluted in immunobuffer. Final dilutions in 20ml PBS, 300μl normal goat serum, 66μl antibody to give 1:300 dilution were made before use

Trizma Base: Trizma base was obtained from Sigma. 6.06g trizma base was added to 950ml distilled water, 4.5 ml of HCl added and the pH set to 7.4.

DAB kit: DAB kit (containing 100µl buffer, 200µl DAB, 80µl hydrogen peroxide and 80µl nickel solution) was obtained from Vector laboratories (UK). 6 drops of buffer stock solution, 20 drops of DAB, and 10 drops of peroxide were added to 15ml of distilled water.

ABC: ABC (actin-biotin complex) was obtained from Vector laboratories (UK). 2 drops of actin and 2 drops of biotin were added to 5ml PBS and left to stand for 30 mins before use, at room temperature.

PBS: PBS (Phosphate buffered saline) consisted of 0.01M phosphate buffer, 0.0027M KCl, 0.137M NaCl and saline. pH was buffered at 7.4. All components were obtained from Sigma

Immunobuffer: Immunobuffer consisted of NaCl (7g), KCl (0.37g), Na_2HPO_4, (1.21g), NaH_2PO_4 (1.80g), trizma base (1.2g), Thimersal (0.4g) and Triton X-100 (3ml) in 1 litre of distilled water. pH was between 7.4 and 7.6 (adjusted with NaH_2PO_4). All component were obtained from Sigma.

7.1.4 Study 2

Butaprost: Butaprost was obtained from Sigma. It was dissolved in ethanol to a concentration of 100µg/100µl, frozen as aliquots at $^{-}70^{o}C$ and further diluted in saline before use.

EP antagonists: GW627368 and ZM325802, were a kind gift from Ged Giblin, GSK.

Both compounds were dissolved in 66% PEG 200, 1% DMSO and 33%H_2O

Naratriptan: Naratriptan was donated by GSK. It was diluted in sterile saline, however to ensure adequate solvency, the solution was run under hot water.

PGE_2: PGE_2 was obtained from Sigma-Aldrich (UK), dissolved in ethanol and saline and frozen as 3mM aliquots. Subsequent dilutions were made in 0.9% saline to a final concentration of 1mM in 1% ethanol and 99% saline.

7.1.5 Study 3

MO: MO (Mustard Oil or Allyl isothiocyanate) was obtained from Sigma. Because of the hazards associated with it, use was dictated by a risk assessment form. For use it was diluted to 10% in mineral oil.

DMSO: DMSO (Dimethyl sulfoxide) was obtained from Sigma.

Glucose saline: Glucose saline (4% glucose by volume) was obtained from Aquapharm

Mineral Oil: Mineral oil was obtained from Sigma.

PEG_{200}: PEG (Polyethylene glycol) was obtained from Sigma.

Sterile saline: Sterile saline was obtained from Phoenix Pharma Ltd Gloucester, UK

Sterile water: Sterile water was obtained from Arnolds Vetinary Products Ltd. UK

Tween 80: Tween 80 was obtained from Sigma

7.2 Appendix B Analysis of Electrophysiological data

7.2.1 Sequence files

To ensure accuracy and consistency of data collected during in vivo electrophysiological recordings, every effort was made to automate stimulus evoked recordings. This was done by using sequence files derived with spike 2 software to drive the stimulator, an example of a basic programme to drive trains of 50 electrical stimuli in cycles of 5 minutes is shown below

7.2.1.1 50 x 1Hz

SET 5.00 1 0	This defines the clock rate, in this case 200 ticks =1 second
HALT	Holds the running of the programme until it' manually started via the PC
'y CALL4 start,0	
START DAC2 5.0 DELAY 10 DAC2 0.0	Drives the 1401 to send a TTL signal to the DAT and switch on recording
DELAY 1000	Delays the programme by 5 seconds
CALL1 es,50	Defines the number of times the programme cycles before pausing, in this case, 50 times

ES:	DAC0 5.0 DAC0 0.0	Drives the 1401 to send a TTL signal to the stimulus isolator to generate electrical stimulation defined by the Neurolog.
	DELAY 200	Delay =1 second
	DBNZ1 es	Returns the cycle to CALL1 es,50 and calculates how many cycles are left (i.e. 50-1)
	DELAY 1000	Delay=5 seconds
	DAC2 5.0 DELAY 10 DAC2 0.0	Switchs DAT recording off
	DELAY 50000	Delay=250 seconds
	DBNZ4 start	Returns the programme to start
'z	HALT	Ends the programme

7.2.2 On line analysis of neuronal responses

During recording of data it was essential to analyse neuronal responses to ensure reproducibility. In the first case this was done by the use of script files derived from spike 2 software, an example of which is shown below;

7.2.2.1 Script file

var t:=0;	start of experiment
var g:=0;	Defines which stimulation number within train data is from
var b%;	count (l, s, q)
var a%;	count (l, u, l)
var h%;	Defines evoked activity
var p%;	Defines spontaneous activity
var z%;	Defines total activity
var y%;	Defines the number of stimuli left in the train to analyse after cycling
var w%;	spontaneous events in 800ms
var w;	Definition of where analysis ends
var l;	Defines the period of analysis after the stimulation
var x;	Definition of number of stimuli in trains
var v;	Definition of where analysis starts
var u;	Defines the period between stimulation

	and the start of analysis
var q;	Defines where the period of pre-stimulus analysis should end (this was at least 5ms before the start of the stimulus in all script files)
var y;	Definition of analysis period before stimulus
var s;	Defines where the period of pre-stimulus analysis should start
var m;	Definition of the factor to average spontaneous data

View (LogHandle()). EditSelectAll(); View (LogHandle()). EditClear();	Controls the output window where results are displayed

PrintLog ("Train Spont Total Evoked\n\n");	Data titles

y:=Input ("Pre-stimulus duration", 0.05, 0, 250);	Allows definition of the duration of analysis before the stimulus

v:=Input("Post-stimulus duration, from?", 0.00, 0, 10);	Allows definition of where the analysis starts from

w:=Input("Post-stimulus duration, to?", 0.05, 0, 10);	Definition of where analysis

ends

x:=Input ("Number of stimuli per train?", 50, 1, 100); Defines number of stimuli in a train

m:= (0.8/y); Defines the nearest real number to enable averaging of calculated spontaneous events in a 800ms period for comparison with defined period of pre-stimulation

repeat — Start cycling between here and "next" command

w%:=0;

z%:=0;

for y%:= x to 1 step -1 do — Decreases the stimulus train number by 1 on each cycle until 0 then allow the programme to move on.

t:= NextTime(32, t);

l:=t + w; — Definition of the bins to be counted

u:=t + v;

s:=t - 0.805;

q:=t - 0.005;

a%:=Count(1, u, l); Counts events on channel 1 after the stimulus defined by v and w

b%:=Count(1, s, q);	counts events on channel 1 in the 800ms preceding the stimulus in the defined period of s and q (-5ms and –805ms)
w%:=w%+b%;	Calculates the corrected number of spontaneous events in the 800ms preceding the stimulus
z%:=z%+a%;	Calculates the number of events in the defined post-stimulus time
next;	
p%:=round (w%/m);	Calculates the number of spontaneous events in the 15ms by averaging the events in 800ms calculated above
g:=g+1;	Adds data from next stimulation response
h%:=z%-p%;	evoked activity = total activity minus spontaneous activity
PrintLog("%4d\t%4d\t%4d\t%4d\n", g, p%, z%, h%);	formats the data into columns defined by g, p%, z%, h%
until t=MaxTime(32);	Moves the analysis programme on to the next stimulation train
FrontView (LogHandle());	Displays the data in a window at the front of the view
FileSaveAs ("?.txt",1);	Option to save data

7.3 Appendix C Manufacturers and suppliers

All equipment not listed below was kindly made by the engineering department at GlaxoSmithKline.

Equipment	Supplier
Stereotaxic frame	Kopf, USA
CED 1401 plus	Cambridge Electronic Design, Cambridge, UK
Rat ventilator	Harvard apparatus Ltd. Edenbridge, Kent
Micromanipulator (model FMC100)	Newport Corporation, Irvine CA, USA
Syringe driver	Harvard apparatus Ltd. Edenbridge, Kent
Headstage	Digitimer, Herts, UK
Oscilloscope	Harvard apparatus Ltd. Edenbridge, Kent
Tungsten recording electrodes	World Precision Instruments, Stevenage, Herts, UK
Bipolar stimulating electrodes	Harvard apparatus Ltd. Edenbridge, Kent
Homoeothermic blanket unit	Harvard apparatus Ltd. Edenbridge, Kent
Neurolog components	Digitimer, Herts, UK
Dental drill	Foredom Electric Co., Bethel, USA
Spike 2	Cambridge Electronic Design, Cambridge, UK
Video capture card	Hauppage, U.K.
Video dimension analyser system & Notocord data capture system	Living Systems Instrumentation, USA
DAT recorder	Bio-logic science instruments, France
Cannula	Portex, UK

Intravital Microscope	Moritex Europe Ltd

Table 14 Manufacturers and Suppliers of equipment used in these studies

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